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# **The Cytoplasmic Inclusions in the Spermatogenesis of Man**

By

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and

**H. W. Beams,**

State University of Iowa.

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With Plates 1-4, and 1 Text-figure.

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## **INTRODUCTION.**

It has been evident for many years that a correct description of the cytoplasmic changes leading to the formation of the human spermatozoon has been needed. We have endeavoured to fill in this gap in the present paper. We cannot claim that the work is completely finished, but most of the outstanding difficulties have been explained, at least, according to our views. We have tried to give clear figures which might be useful for histology and anatomy text-books, instead of the obsolete ones now found in such manuals, and in the text we have provided complete and separate descriptions of the plates, such as would be suitable for using in toto as text script in these manuals. We have adopted the simplest nomenclature, and have based this on the description of the adult sperm shown in fig. 14, Pl. 3.

The preparations were all made by H. W. Beams and jointly studied by both of us, and the figures embodied in this paper have been drawn not only to show the actual appearance of each stage (Pls. 1 and 4), but also our interpretation of some doubtful points in the spermatogenesis (Pls. 2 and 3).

The set of preparations we had to study, one of us (J. B. Gatenby) believes to be as perfect as modern technique makes possible. Unfortunately, however, perfect preparations do not

make the spermatids any bigger than they are, and they are really very small objects to study. On the problem of the centrioles we cannot claim, perhaps, to have had better material than, for instance, Meves or Branca; but in our material the cytoplasmic inclusions are demonstrated in a manner not bettered by the usual material made after repeated trials from the laboratory mammals. Unfortunately the Da Fano material was uniformly unsuccessful for reasons unknown to us.

In fig. 14, Pl. 3, we have given our view of the structure of the adult human spermatozoon. The nucleus is covered by two caps, a front one (A.) or acrosome, and a hind one (PNC.) or post-nuclear cap. In the human, but not always in other spermatozoa, the two caps meet completely at the border (B.). Now this head is attached to the middle-piece ( $c^1$  to  $c^2$ ) by a neck (NK.) in which a single structure (NB.) or a more complicated system of neck granules exists. We believe that this is not a true centriole region, but is only connected to the first centriole ( $c^1$ ), and that in the human sperm the head centriole ( $c^1$ ) does not divide. The mitochondria (M.) are plastered on a skeleton (MPS.) which is a cytoplasmic sheath around or formed from the original axial filament. The second, or ring, centriole ( $c^2$ ) lies at the lowermost part of the middle-piece. We have been unable to find any definite spiral structure at the middle-piece, or any other bodies in the head, neck, and middle-piece regions of the human spermatozoon.

This paper, therefore, attempts to give an account of the formation of the acrosome cap, post-nuclear cap, neck, centrioles, middle-piece, and flagellum in the human spermatozoon.

We wish to acknowledge the interest which the following have shown in the scientific problems involved in this work: Dr. H. L. Snyder, Mr. Wylie Cook, Dr. Howard E. Snyder and Dr. Cecil D. Snyder of Winfield, Kansas, and Dr. H. E. Branch of Wichita, Kansas.

#### METHODS.

Ether was used as an anaesthetic. Material was cut into small pieces and fixed in less than one minute after the blood-supply had been interrupted. Specimens from six individuals

were used, the ages being 15, 16, 18, 19, 25, and 26 years. The methods tried were those of Kolatschew, Mann-Kopsch (Weigl), Da Fano, Champy, Zenker, Helly, Regaud, Bouin, Allen B 15, cold Flemming, Carnoy (Le Brun). The stains used were Feulgen, iron alum haematoxylin, and acid fuchsin. The most beautiful preparations were Kolatschew and Weigl sections treated in permanganate of potash and oxalic acid and then stained by iron alum haematoxylin or Benda's alizarin and crystal violet. In the latter the Golgi apparatus was black, the mitochondria violet, and nuclear structures yellowish-brown or purplish-brown. In the Kolatschew and Weigl iron alum haematoxylin slides the Golgi apparatus was black, and the mitochondria blue-grey to blue-black, according to differentiation. So far as we are aware this is the first time these methods have been used for a study of mammalian spermatogenesis. In fig. 31, Pl. 4, is a drawing of the old Benda method, in figs. 26, 28, and 31, Pl. 4, of the new combined method.

In 1921 one of us tried to reproduce the methods recommended by Papanicolaou and Stockard for staining the proacrosomic granules bright red in acid fuchsin. At that time it was not found possible to make this special acid fuchsin method work properly, and it was with some curiosity that one of us saw it tried again by H. W. Beams. Just as on the previous occasion no successful preparations of the type figured by Papanicolaou and Stockard were forthcoming, and we are inclined to believe that these authors either had a very different specimen of acid fuchsin from those we have tried, or else have not given a clear description of their methods. In both cases one could say that weak staining with acid fuchsin produced a diffuse, pinkish-red section, or stronger staining tinged bodies which might just as well have been the remains of the mitochondria.

The lens used was a Beck  $\frac{1}{4}$  inch (1.8 mm.) on a Spencer binocular microscope with eyepieces  $10\times$  and  $6\times$ . The figures in Pls. 1, 2, and 4 were made with the aid of a camera lucida modified to fix on to the sloped binocular tubes, and the cells depicted were post-osmicated and therefore larger than in Bouin material, in which shrinkage is greater.



## LITERATURE.

There is, of course, an enormous literature on the cytoplasmic bodies in the spermatogenesis of mammals, most of it dealing with the guinea-pig or rat. There is a good number of papers on the human, the most recent being that of Gatenby (9) on staining human spermatocytes in neutral red. There is a very large paper on human spermatogenesis by Branca (4) and, of course, the well-known papers by von Winiwarter (29), Painter (23), and Evans and Swezy (6) on human chromosomes, with which we do not deal. The whole subject of the formation of the mammalian spermatozoon goes back to the spacious days of Benda, Retzius, and Meves, and their more recent successors, Duesberg and Regaud. Latterly, Papanicolaou and Stockard (23), and Gatenby and Woodger (13) have made contributions to the subject. Robert Bowen (1), while not having the opportunity of studying human spermatogenesis, drew up some figures illustrating what he thought occurred during the formation of the human sperm. His figures were based on the studies of Meves, Branca, Benda, Retzius, and Gatenby on the human and other mammals. As a matter of fact, this survey by Bowen has interested us very much, as well as his trenchant comments on the outstanding problems with which he was confronted when trying to draw up a scheme of human spermatogenesis which might be considered correct. He says, 'I am so disgusted with the frightful figures of human sperm formation so universal in medical texts, that I spent rather more care than otherwise in the production of a series that might be more acceptable. Much of it is, of course, added from our knowledge of other mammals, but this seems justifiable and in any case the errors are nothing to compare with the ones current in present figures.'

Bowen's interpretation of the centrioles in human spermatogenesis is that of Meves, and is partly illustrated in figs. 23-5, Pl. 3. On the question of the centrioles he explains that he has found the whole matter in a state of confusion, and in this, as in other parts of his scheme, he is merely giving his interpretation of the figures of other people. He begins with two centrioles as in fig. 23, Pl. 3, the flagellum having broken out

from the distal centriole (DC.)—the proximal being free. Between the stages of figs. 23 and 24, Pl. 3, the proximal has passed up and become stuck on to the nucleus to form a flat body, and the distal has divided into two, a ring and a round centriole (DC<sup>1</sup>, DC<sup>2</sup>). As will be mentioned below, we disagree with all of this interpretation.

Regarding the acrosome, we agree with fig. 23, Pl. 3, but consider figs. 24 and 25, Pl. 3, are incorrect, in so far as part of the acrosome is supposed to be formed directly from the acrosome vesicle (marked A. in fig. 23, Pl. 3).

Regarding the Golgi-idiozome complex, Bowen says, 'My representation of the Golgi rodlets plus idiosome is the one usually given, particularly following Gatenby. I think it is probably an inadequate picture of the true situation and that in ten years from now, in another revision, you would want to correct it.'

As will be seen in this paper we have found no reason for altering the current description, the Golgi-idiozome complex in the human being just like that of any of the laboratory mammals studied in recent years.

Bowen draws the mitochondria granular—this is correct, and they are certainly not chondriomites. Regarding the middle-piece, Bowen accepts the idea of a mitochondrial spiral, but the description of Meves current in certain English anatomy manuals (5) does not mention a mitochondrial spiral. There is the spiral sheath of Meves, but the mitochondrial material is outside this and Meves' spiral sheath is of ground-cytoplasmic rather than mitochondrial formation. There are, of course, mitochondrial spirals on some mammalian spermatozoa. In any case, we feel very dubious about some of Meves' ideas on the structure of the fully formed spermatozoon.

It will be noticed, when this paper has been read, that we differ from Bowen's interpretations on the following points:

- (a) The exact constitution of the acrosome.
- (b) Dictyokinesis.
- (c) The centriolar apparatus.
- (d) The middle-piece.

As a matter of fact, the dictyokinesis is somewhat different from

what we ourselves had expected, and the arrangement of the centriolar apparatus could still be regarded as a matter of doubt. The confusion, as we think we can show, has been due to the fact that the older workers never realized that the centrioles are not the only bodies in this region of the forming spermatozoon. We never found a spiral structure in the middle-piece region of the human spermatozoon, and the acrosome appears to us to be formed of one substance. The neck and upper centriole region in mammalian sperms is still ill understood.

In Petrogale, however, Vejdovsky clearly describes the following parts in the spermatid—mitochondria, a mitotic apparatus (Golgi apparatus of modern workers), two centrioles, and the rudiment of the neck body or granules (RNB.). This rudiment (fig. 19, Pl. 3) develops later into three knots (figs. 20 and 21, Pl. 3) and a hyaline, intermediate substance forming inter-connecting bridges (or centrodemeses). Vejdovsky (27) says: 'The rudiment formed by the said three knots and the hyaline intermediate substance, representing the neck of the later spermatozoon, are present already from the very beginning of the spermatids, and in the further development they adapt themselves to the head and to the axial filament. In our further description, we shall distinguish the two centrioles at the back as proximal ( $c^1$ ) and distal ( $c^2$ ); the three knots in front connected by the centrodemeses with the proximal centriole will be called neck. It does not appear in all stages as it is often pushed into the nuclear contents and covered by it.'

Examination of the figures of mammalian spermatids by many authors will show that this arrangement of knots in the neck region is very common. Unlike Vejdovsky, these authors have not realized that the knots and the centrioles are different. Moreover, as pointed out elsewhere in the spermatids of many other lower animals, the upper centriole is accompanied by another body, the so-called post-nuclear body of Gatenby, which we now regard as a neck granule, in every way homologous with Vejdovsky's neck rudiment.

Some years ago one of us (12) found that in Da Fano silver preparations of guinea-pig testis, the ripening sperms had a remarkable chocolate or black band as shown in fig. 18, Pl. 3,

PNC. These bands were so clear that they were easily photographed (see 'Proc. Roy. Soc.', B, vol. 104, p. 473). Of their presence there can be no doubt, and in figs. 15-17, Pl. 3, we have given some drawings of the formation and evolution of this structure in the spermatids of *Cavia*. In favourable cells the band may be seen to originate from some granules which become grouped behind the spermatid nucleus at the time of fixation of the centriole. Between the stages in figs. 17 and 18, Pl. 3, the acrosome (A.) extends down the side of the sperm head and meets the post-nuclear cap, but leaves a part of the nucleus bare (BN.).

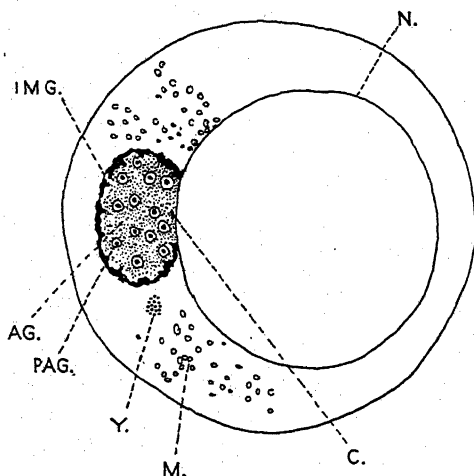
AVOIDANCE OF CONFUSION BETWEEN THE NECK GRANULE,  
PLATE, OR KNOTS, AND THE POST-NUCLEAR BODY  
OF GATENBY (12).

One of us recently homologized a number of bodies in the hind region of the head of the animal spermatozoon. It should be carefully noticed that most of these bodies mentioned by Gatenby are more likely to be the homologues of the neck granules, rather than the argentophile post-nuclear body of the guinea-pig spermatozoon. We have, therefore, retained the words 'post-nuclear cap' for the hind cap of the human spermatozoon, and have used the current term 'neck, knot or body' for the neck structures. This introduces no new terms into this paper, and it does not raise the question of the exact homology of the various bodies mentioned by Gatenby in his previous paper (12). In figs. 15-17, Pl. 3, this description is made clear. The neck apparatus begins as a rudiment in fig. 15 (NB.), Pl. 3, quite separate from centrioles ( $c^1$  and  $c^2$ ) and from the fore-runners of the post-nuclear cap (PNC.). In fig. 16, Pl. 3, the granules and centrodesmoses (NB.) of Vejdovsky have appeared and have become related to the undivided proximal centriole ( $c^1$ ).

CELL NOMENCLATURE.

In recent years so many new and unwanted terms (10, 23) have been introduced into this type of cytology that it is necessary carefully to avoid using them. In Text-fig. 1 is a spermatocyte which will illustrate the nomenclature we have

adopted. Outside the nucleus and lying on it is a somewhat complicated body referred to by the older workers as the sphere, idiozome, or archoplasm (archiplasm). The older workers knew that within this body were minute vacuoles containing granules which later formed part of the acrosome, or the whole acrosome.



TEXT-FIG. 1.

Spermatocyte to illustrate nomenclature used. IMG., impregnating (chromophile) part of Golgi apparatus; AG., chromophobe part of Golgi apparatus (idiozome, archoplasm); PAG., pro-acrosomic granules, or forerunners of spermatid acrosome; C., centriole; Y., Y-granules or rubrophile granula; M., mitochondria; N., nucleus.

In recent years it has been shown that this sphere has an argento-philic or osmiophilic cortex. Nowadays we refer to the sphere and its cortex as respectively the chromophobe (AG.) and chromophile (IMG.) parts of the Golgi apparatus. Since the granules within the sphere later form the acrosome they have been called the pro-acrosomic granules (PAG.).

In the cytoplasm scattered here and there are many mitochondria (M.). These are globular and not rod-like. In addition is a group of granules (Y.) which has been described in *Saccocirrus* fixed by chrome-osmium methods (7). These granules as shown by Hirschler (15) stain brilliantly in neutral red intra

vitam, and we will call them Y-granules<sup>1</sup> following MacBride and Hewer (18).

All these parts can be seen intra vitam and without staining in most animal germ-cells (8, 10).

#### THE HUMAN SPERMATOCYTE STAINED SUPRA-VITALLY.

In 1930 one of us (9) had the opportunity of studying some neutral-red-stained spermatocytes from a man 43 years of age, who had been operated upon for a hernia. The Golgi apparatus could be seen faintly in the living cell, and the most conspicuous elements which stained brightly in neutral red were firstly the Y-granules (rubrophile granula) of Jan Hirschler (15), shown in fig. 22, *v.*, Pl. 3, and secondly a peculiar vacuolated structure, *x*. Besides these bodies there often occurred a group of tiny needle- or baton-shaped crystalloids (*xc.*) always lying near the Y-granules. We are unable to say whether these crystalloids are or are not neutral red dye crystals, or the same type of body as the Lubarsch, Charcot-Böttcher, and Reinke crystalloids. In guinea-pig testis stained similarly in neutral red, one of us has never found crystals, and we incline to the opinion that the crystalloids are not neutral red artifacts. In view of this amount of doubt we have not put any crystals in the spermatocytes in our scheme on Pl. 2.

The body marked *x*. in fig. 22, Pl. 3, is another enigmatic structure. We believe that a similar accessory body may be present in our Weigl and Kolatschew slides which form the basis for the description of human spermatogenesis given in this present paper. There seems to be an extra body besides the Golgi bodies in many spermatocytes in Weigl slides, but this could also be the impregnated Y-granules, or unassembled Golgi material, so we cannot express any definite opinion. This extra body is depicted in fig. 4, *ABX.*, Pl. 2, and fig. 1, Pl. 1, middle.

<sup>1</sup> These granules have recently been studied by J. A. Muliyl, who finds that they stain best in Nile Blue (deep blue colour), come and go according to whether the animal is fed or starved, and exactly resemble the first formed yolk in young oocytes. From Muliyl's work it seems that the Y-granules are abortive yolk, and chiefly fatty acid. They can be seen in the living germ cells of many animals.

It should be mentioned that we have never seen human spermatids stained in neutral red, so we cannot say whether they contain Y-granules. Since all known spermatocytes in mammals, insects, annelids, &c., which contain Y-granules also have them in their spermatids, we have ventured to put them into our semi-diagrammatic figures in Pl. 2.

#### THE GENERAL APPEARANCE OF SECTIONS OF THE HUMAN TESTIS.

Examination of the ordinary chrome-osmium preparation of the human testis shows that much more fatty material is present than in any of the usual animals studied, such as rat, mouse, or guinea-pig. This fatty substance is found in the Sertoli cells, and is mainly situated in the base of the cells (fig. 1, Pl. 1). It goes yellow or yellowish-brown in chrome-osmium. The next most striking fact is the presence of numbers of peculiar crystals, which are found in Sertoli cells, interstitial cells, and spermatogonia (fig. 1, Pl. 1). The person only acquainted with the spermatogenesis of the laboratory animals will immediately notice that in the human the Golgi apparatus of growing and full-grown spermatocytes is often in the form of more than one aggregation (fig. 3, Pl. 2). Another peculiarity is the almost constant presence of a vesicle or vacuole in the developing and developed heads of the spermatozoa (figs. 12, 13, x., Pl. 2).

In fig. 1, Pl. 1, is a part of an active spermatid tubule, as well as two interstitial cells (above). Below the interstitial cells and on each side are two Sertoli cells, the one on the left being complete and showing how these cells stretch right out to the lumen and provide a nidus for the heads of the developing spermatozoa. In these cells the fat is shown at the base of each cell and, as has been remarked, attracts attention immediately. Just alongside the Sertoli cells are the spermatogonia. These are watery cells with ovoid nuclei, the cytoplasm being often very large in comparison to the nucleus. Occasionally crystals of a rod-like nature occur in these cells, as shown in the spermatogonium on the left. Such spermatogonia bud off cells inwardly by mitosis, and these grow into spermatocytes. Four spermatocytes at various stages are shown in the diagram.

Between the spermatogonia and spermatocytes, but not so clearly between the spermatids, are remarkable intercellular spaces which can be traced mainly as parts of Sertoli cells. The spermatocytes, when full grown, divide to form spermatids (bottom right) which metamorphose into spermatozoa (middle bottom and bottom left of diagram). It may be mentioned that in fig. 1, Pl. 1, the spermatogonia are of a size in normal proportion to the nuclei of the spermatocytes, whereas in the scheme in Pl. 2 the Sertoli cell and spermatogonium shown are smaller than normal, though equally small examples can be found in proportion to the size of the spermatocyte in fig. 4, Pl. 2. In addition, whereas in fig. 1, Pl. 1, the ripe sperms are drawn in proportion to the other cells, in figs. 10-13, Pl. 2, these stages are rather larger than usual, in proportion to the spermatocyte in fig. 4, Pl. 2. This has been done for the sake of making the diagrams plainer.

#### THE SERTOLI CELL.

These are beautiful cells in the human testis. The base of the cell rests against the connective tissue-wall of the tubule, and the cytoplasm stretches outwards in a varying degree according to the type of cell the Sertoli cell is serving. Many pyramidal, short Sertoli cells exist, but when the Sertoli cell is serving as a nurse for the developing spermatozoa it stretches a long way towards the lumen, as shown on the left in fig. 1, Pl. 1. Now the granules of fat in the Sertoli cell are almost always located in the base of the cell, and consist of spheres with various degrees of staining in osmic acid. Some go black, but mostly they are yellowish, and many contain a vacuole which does not stain.

The nucleus often contains intra-nuclear folds, which are present in other types of cells. One such fold is shown in the Sertoli cell on the left of fig. 1, Pl. 1. The Golgi apparatus is of a slightly branched filamentary type, and is remarkably clear in good preparations. It is interesting here to note that there are three distinct types of Golgi apparatus in the human testis—the filamentary type (Sertoli cell), the sub-spherical type (spermatocyte), and the semi-dispersed type (spermatogonia).



The mitochondria in the Sertoli cell lie principally between the Golgi apparatus and the fat granules, and are granular in nature.

Careful examination of the spermatocytes shows that intercellular spaces are very marked in some cases (fig. 1, Pl. 1, intercellular spaces, upper). These spaces have been noted by other workers, such as Vejdovsky, and undoubtedly in many cases are directly in communication with the Sertoli cells. That is to say, the spermatogenetic cells actually lie partly inside the Sertoli cells, surrounded by a protoplasmic intercellular substance, which is provided with fatty and other granules. In our preparations of the 'Native Cat' (*Dasyurus*) a similar appearance is present. We have never found anything quite so striking in the guinea-pig or rat, though a type of 'nutritive syncytium' does exist in both of these animals.

#### THE SPERMATOGONIUM.

The spermatogonia do not usually contain crystalloids, but when these are present they are rod-shaped rather than needle-shaped. Unless well fixed, and even sometimes when surrounding cells are apparently well fixed, the cytoplasm of the spermatogonium is found to have collapsed and left numerous vacuolated regions. It is certain that the spermatogonial protoplasm is more watery than that of the spermatocytes and older categories of cells. In Weigl and Kolatschew slides the Golgi apparatus is found to be formed of a cap over one end of the nucleus. The cap is constituted by numerous blocks of osmophile material, and in the majority of cases no discrete or segregated apparatus may be said to be present. Later, as we shall see, the blocks do run together to form a segregated apparatus.

Mainly near the Golgi apparatus, but further out in the cytoplasm, are found numerous minute spherical mitochondria. No fat or yolk has been found in such cells, but sometimes a crystalloid—the so-called Lubarsch crystal—does occur. A typical one has been put in the spermatogonium on the left middle of fig. 1 and in fig. 2, Pl. 2 (4). Previously one of us (9) had expressed doubts as to the theory of the Sertoli cell-determination through the medium of a crystalloid, and both the present authors are agreed that this theory has little to support it.

## THE SPERMATOCYTE.

As spermatogonium grows into spermatocyte the cytoplasm becomes less definitely watery and more resistant to fixation. The mitochondria spread out completely and form a dense area around the nucleus. The Golgi apparatus begins to run together to form one or more large discrete areas (fig. 3, Pl. 2) which in most cases ultimately come to form one single body as in fig. 4, Pl. 2. Striking examples may be found, however, of spermatocytes with one large Golgi apparatus and an accessory Golgi body nearby; at first we considered that such examples had one large Golgi apparatus, and that the accessory body was of a different nature. Gatenby (9) had already described a peculiar body (fig. 22, x., Pl. 3) which stained red in neutral red, and which, in many cases, would be about the same size as the accessory body in the Kolatschew prepared spermatocytes. While we incline to the view that many of these accessory bodies are merely parts of the spermatogonial Golgi elements which have failed completely to assemble together, there is still the possibility that we are dealing with something quite different. In fig. 1, Pl. 1 (middle-right), is a spermatocyte which shows the type of thing we have mentioned.

Now the structure of the Golgi apparatus is exactly like that found in other mammals such as the guinea-pig or rat. That is to say, with Carnoy, Bouin, corrosive acetic fixation, followed by such staining as alum haematoxylin, a dense area of cytoplasm is revealed in this region and is referred to generally as the sphere, archoplasm, or idiozome. In preparations made by Champy's fluid and Benda's crystal violet, minute vacuoles containing beads may be made out in favourable cells. Such a spermatocyte is shown in fig. 31, Pl. 4, the archoplasm staining from yellowish to violet, usually more violet than yellow. The tiny beads inside the vacuoles, in favourable examples, are deep violet. They are extremely small and can be found only after a good deal of searching. Now these granules have been described by many of the past masters of cytology, especially by Moore, Meves, Benda, and Niessing (see 13). It is widely believed (and we believe it ourselves) that

these granules are the actual forerunners of the acrosome bead which in later stages of the sperm formation is deposited on the spermatid nucleus (fig. 27, Pl. 4) and forms the head cap of the spermatozoon. We will call these pro-acrosomic granules. Now by the Golgi apparatus methods a cortex of osmiophile, or argentophile, material is found in the sphere or idiozome, and together these form the Golgi apparatus. Our material by the Kolatschew and Weigl methods is particularly beautiful, and offers excellent opportunities for studying the condition of the cortex in different examples. Sometimes the osmiophile material is smooth, sometimes crenated, sometimes raised into beads, and sometimes even batonette-like in arrangement. The Golgi apparatus is jet-black and by far the most conspicuous part of the cell in Benda-Kolatschew and such preparations.

With the exception of the somewhat doubtful crystalloids shown in fig. 22, Pl. 3, and the peculiar method of assembly of parts of the Golgi apparatus, the cytoplasmic inclusions of the human spermatocyte do not appear to be different from those of other animals.

#### DICTYOKINESIS.

The dictyokinesis was of a somewhat unexpected type. For one thing, in many of the cells the Golgi apparatus broke up into much finer granules than is the case in the rat or the guinea-pig. In some cells the granules were so fine as to form a dark cloud rather than a visibly granular area. The main steps in the process are as follows. During the early prophase the idiozome or archoplasm breaks up, and the osmiophile cortex appears to separate into a cloud of granules like a rising sun. These pass further and further into the cytoplasm till pieces of osmiophile material are to be seen through the main body of the cell, but not so much at the poles. In fig. 5, Pl. 2, the metaphase stage is shown, with Golgi material (G.A.) grouped a little more near the inside of the asters than elsewhere. This is followed by stages which end up with a grouping of Golgi material as shown in fig. 33, Pl. 4. Here the arrangement is rather peculiar, and six examples of this stage have been examined, the figure given in Pl. 4 being typical. The poles

of the cell are practically always free of osmiophile material or, mitochondria, the Golgi material being grouped on the inside of the spindle in the contour of the fused chromosomes of telophase. Thus there is a grouping of Golgi material not, as in many examples of dictyokinesis, about the asters, but rather around the nuclei. This leaves two daughter spermatids with a scattered Golgi apparatus consisting of a number of different-sized elements scattered a little more to one side of the cell than to the other, as shown in fig. 7, Pl. 2, and fig. 32, Pl. 4, the latter being a more advanced stage. Very soon, however, all the parts fuse and form a single Golgi apparatus, as in fig. 8, Pl. 2.

#### THE CENTRIOLES.

The centriole appears first clearly in the resting-stages of the spermatocytes, although it may be found in the mitoses of spermatogonia. In the spermatocyte it is usually found between the nucleus and the Golgi apparatus (fig. 31, Pl. 4), sometimes slightly embedded in the latter. By the end of the growth stage of the spermatocyte it is usually diploid. In the spermatid, as has already been indicated, it has no apparent direct connexion with the assembly of the fragmented Golgi apparatus, and is found near the edge of the cell, as shown in fig. 7, Pl. 2. It passes to the surface of the cell, on the way dividing into two equal parts. It rests under the cell-wall, and a flagellum grows out jointly from both bodies. At this stage the centrioles are distinctly unequal in size, adhere closely, and the moiety nearest the cell-wall becomes ring-shaped, and subsequently continues to grow until it is relatively very large compared with the head centriole—as it may be called (fig. 8, Pl. 2). Subsequently the centrioles, both in contact with the flagellum and keeping closely together, move inwards and become attached to the side of the nucleus opposite to that upon which the acrosome bead is being deposited (fig. 9, Pl. 2). The ring centriole is now a very conspicuous structure, and continues so till the last stages of sperm formation. This centriole keeps its position just behind the nucleus and the head centriole until the sperm head is nearly fully formed (fig. 11, Pl. 2), but then the ring slips

down the axial filament and takes up its position some distance below, as shown in fig. 12, Pl. 2, and figs. 26 and 30, Pl. 4. Here it stops and subsequently becomes smaller and less clearly stainable. Eventually it forms the bottom of the middle-piece (fig. 13, Pl. 2, and fig. 29, Pl. 4).

We may now turn to the head centriole. As has been mentioned, there is considerable diversity of opinion as to what happens here. We ourselves hold the view that in the majority of cases nothing happens, and the head centriole from the first is the *fons et origo* of the flagellum and never becomes separated from it. This part of the spermatozoon is very difficult to study owing to the fact that the head is now so small, and methods which stain the centrioles sharply also tend to stain the nucleus equally sharply at this period. We have, in a few cases, found two bodies quite distinctly in this region, as shown in fig. 29, x., c<sup>1</sup>, Pl. 4, and believe that the upper body is either a second structure distinct from the head centriole or else a special thickening of the nuclear or other membrane at this point. In the works of other authors, such as Branca (4), an additional bud-like or flat body may be shown attached to the head centriole, and in some cases a very complicated arrangement of granules is figured here.

In fig. 9 (NBX.), Pl. 2, we have drawn this small rod-like structure which appears to project out just near the head centriole. We have never found it in Champy-Benda sections which show the true centrioles so remarkably, but it is common in Zenker iron haematoxylin slides. In other words, we regard it as of non-centriolar nature. We feel that it might be the same as the neck-body rudiment of Vejdovsky, figs. 19-21, Pl. 3, and in a few cases we believe that we have found it separate from the centrioles in the earlier spermatid. As we have mentioned, Branca especially often figures such a structure, and it is largely a matter of interpretation as to what it is. One view, which is attractive, is that it is part of the future neck apparatus of the adult spermatozoon, and later becomes associated with the undivided head centriole, either as a flat plate or as a number of granules. We do not accept the view that this body (NBX.) is a bud from the head centriole.

## THE ACROSOME.

This is very clear during the spermatid stages. In the spermatocyte the intra-archoplasmic granules already described and figured in fig. 4, Pl. 2, and fig. 31, Pl. 4, are very probably the forerunners of the spermatid acrosome. We have only found these spermatocyte granules in a small number of preparations, especially those fixed in Champy and stained in Benda's alizarin and crystal violet. They are extremely minute and delicate, and we have certainly been unable to trace them through the spermatocyte divisions as claimed by Stockard and Papanicolaou for *Cavia*. At the same time we consider theirs the correct interpretation. After the Golgi apparatus has assembled in the spermatid (figs. 7 and 8, Pl. 2, and fig. 32, Pl. 4), a minute bead, or sometimes a few beads, can be seen inside the archoplasm after such fixation and staining as Zenker and iron alum haematoxylin or Champy and Benda. In fig. 8, Pl. 2, the clearness of the bead within the Golgi apparatus has been exaggerated. The bead undoubtedly lies within a small vacuole and is soon deposited on the nucleus. We could never get any evidence of the participation of intra-nuclear material in the formation of the acrosome as claimed by Vajdovsky (27). The bead swells after it has been deposited, and the vacuole, now usually semi-lunar in shape, surrounds it, as in fig. 9, Pl. 2, and fig. 27, Pl. 4. The whole arrangement is very conspicuous, and Champy or Kolatschew preparations stained in Benda's alizarin and crystal violet are extremely beautiful and effective for the study of such stages, the nuclei being yellowish, the bead intensely violet. Now the Golgi apparatus detaches itself from the surface of the vacuole and drifts down the side of the nucleus, as shown in fig. 10, Pl. 2, and fig. 28, Pl. 4. The vacuole persists for some time, the Golgi remnant (Bowen), as it may now be called, eventually drifting down into the lengthening-out cytoplasm, as shown in figs. 11, 12, Pl. 2, and figs. 26, 30, and 34, Pl. 4. So far as we are aware, after leaving the nucleus, as in fig. 10, Pl. 2, or fig. 28, Pl. 4, the Golgi apparatus takes no further part in sperm formation. Occasionally it is found apparently wrapped around the bottom

of the spermatid nucleus as it passes down, but we do not believe that this has special significance.

Now soon after the departure of the Golgi apparatus from the top of the nucleus the acrosome bead is found to have spread out to form, or to have become connected with, a laterally situated cap (AL.) which in optical section is shown in fig. 28, Pl. 4. The principal bead (A.) may not at this period have become smaller, but this does not necessarily mean that the lateral cap is not derived from the first-deposited bead. At all events, the newly formed material stains exactly like the first acrosome and is intercontinuous. It should be noticed that the rapid growth of the bead synchronizes with the disappearance of the crescentic vacuole (sp. in fig. 27, Pl. 4). Whether there is any causal relationship between these events we cannot say. As the sperm head goes on metamorphosing the acrosome cap grows down and the first formed bead becomes smaller and smaller until it no longer appears as a bead (figs. 26, 30, Pl. 4). At the stage just before (fig. 34, Pl. 4) the lateral acrosome material appears quite thick. In many spermatozoa, as, for instance, in fig. 11 of Pl. 2, the acrosome cap covers much more than the front half of the spatulate head of the spermatozoon. Eventually the acrosome cap forms the front covering of the ripe sperm head, occupying very little more than the top half of the head, as shown in figs. 12, 13, Pl. 2, and fig. 29, Pl. 4 (in optical section).

Unlike that of the guinea-pig spermatid, the human acrosome does not appear to consist of two separate parts.

#### THE POSTERIOR CAP OF THE SPERMATOZOON.

All spatulate mammalian spermatozoa have a head covering divided into two parts, the anterior cap, or acrosome, and the posterior cap, or post-nuclear cap of Gatenby (fig. 13, A. and PNC., Pl. 2). Now we are quite certain that in the spermatid stage depicted in fig. 26, Pl. 4, there are these two parts, the first being stained violet (crystal violet) and the second black (osmic acid) in Weigl Benda preparations. We have examined many such cells and we are both agreed that this arrangement shown in fig. 26, Pl. 4, is what really exists. In optical section

the appearance is as in fig. 26, Pl. 4, but actually the structure is shaped like a somewhat flattened cup, as shown in figs. 10 and 11, PNC., Pl. 2. Both the origin and the subsequent history of these sharply impregnated structures are somewhat difficult to make out, and we have spent a considerable amount of time both in observing favourable material, and in discussing exactly what we should put in our drawings on Pl. 2. The earliest stage we can trace the rudiment back is shown in fig. 9, Pl. 2, where in many cases a very sharp membrane (PNC.) is found at the back of the nucleus. Between the stages in figs. 9 and 10, Pl. 2, we have studied many examples of this growing membrane, but in other cells it is not demonstrable (fig. 28, Pl. 4). It should be pointed out, however, that we have no Da Fano silver nitrate preparations, and it is in this material that the post-nuclear bodies of *Cavia* are clearest (figs. 15-18, Pl. 3). One of us (J. B. Gatenby) feels convinced that by stage 8 in Pl. 2 the rudiment is already present behind the nucleus, and the condition in fig. 9, Pl. 2, and fig. 10, Pl. 2, is brought about by a creeping up and growth of such material, probably of lipoidal nature. Now so far as the fate of the post-nuclear material shown in black in fig. 26, Pl. 4, is concerned, we believe that it ultimately grows up and joins the acrosome. In figs. 10 and 11, Pl. 2, stages in the growing up are shown, but the acrosome grows down faster, and by fig. 12 in Pl. 2 the two have met. In many spermatids the uncovered space (UCR. in fig. 11, Pl. 2) is very clear, and a condition intermediate between stages 10 and 11, in Pl. 2, is the normal finished condition in the guinea-pig sperm. In Champy-Benda preparations in the same region a space is found alongside the nucleus (sr. in fig. 30, Pl. 4), and we are not sure whether this is the region which goes so black in Weigl and Kolatschew slides, for it may be connected with the manchette, to be mentioned below.

#### THE MANCHETTE.

It seems agreed by most observers that the delicate tube or manchette (figs. 10, 11, MCH., Pl. 2, figs. 30, 34, MCH., Pl. 4) comes and goes without any apparent function. We do not believe that it forms part of the middle-piece, and have no



suggestions of value to bring forward. It has been adequately described by other authors (see, for instance, Meves and Branca).

#### THE HEAD CAVITY OF THE ADULT SPERM.

There appears to be a large cavity or vacuole inside the head of the human spermatozoon (marked v. in fig. 13, Pl. 2, in fig. 29, Pl. 4, and in many other figures). There is also a number of nucleoli in the younger spermatid head, and in some cases the vacuole stains like a nucleolus, but is only doubtfully derived from a nucleolus. We are both agreed that this space is present in the majority of spermatozoa. This space has been figured accurately before, as, for instance, by Retzius (Taf. lxi, fig. 2). We do not know the purpose of the space, but it may be some kind of hydrostatic organ, or even a respiratory vacuole, but we cannot say whether it contains gas or liquid.

#### THE CRYSTALLOIDS.

Crystals, as has been mentioned, are extremely remarkable in the human testis. We cannot add more to what has been described previously (9, 29). The sort of thing we have found is shown in fig. 1, Pl. 1, where there are extremely large, stubby crystals or batonettes in the interstitial cells, smaller rod-like crystals occasionally in the spermatogonia, and needle-like crystals, usually sharp at both ends in the Sertoli cells.

As one of us mentioned before (9) we do not believe that these crystals are Sertoli cell-determiners, and we think they are all modifications of the same material, which is possibly of the nature of storage material.

#### DISCUSSION.

In the case of the large urodele sperm,<sup>1</sup> it has been possible to give a clear account of the formation of the centriole and neck apparatus. In this two centrioles and a neck granule rudiment are implicated, and no division of the head or proximal centriole takes place. This centriole becomes associated with the neck rudiment and does not divide further. There is little

<sup>1</sup> Gatenby, Jour. Morph. and Physiol. v. 51, No. 2. June 5, 1931. See also (12).

doubt in our minds that the confusion in descriptions of mammalian spermatogenesis has been due to the fact that the earlier workers, unlike Vejdovsky, did not understand that the neck granules are not centriolar in nature, but only later become associated with the head centriole.

Like other observers we have found it very difficult to make out exactly what is happening at this stage in human spermatogenesis. It is clear to us that when the filament grows out just after the diploid centriole has come to the surface of the spermatid for that purpose, the outgrowing flagellum originates from both granules, and that these adhere closely together for a considerable time afterwards, certainly until the distal centriole has become ring-shaped. We have never seen a spermatid with one free centriole and another centriole giving rise to a flagellum (Pl. 3, fig. 23). The story of the division of the proximal centriole into two parts and the subsequent fragmentation of one or both of these parts into two or more granules has been developed by Meves, who dominated this branch of cytology up till thirty years ago.

As we have pointed out, there was a flaw in Meves' meticulous descriptions of various vertebrate spermiogeneses—he did not realize that all the granules in the neck region did not necessarily arise from the proximal centrosome. It is greatly to the credit of Vejdovsky that he gave a correct interpretation of the facts in Petrogale.

Until the development of better microscopes and better staining methods, we do not believe that the exact behaviour of the centriolar and neck granules in the human spermatid will be understood more clearly than in the account given in this paper. What is wanted is some form of selective stain for centrioles and neck rudiments, and it seems unlikely that this technique will be forthcoming.<sup>1</sup>

On the subject of the Golgi apparatus there is little to say. It is like that of any other mammal known, and it is not formed

<sup>1</sup> In *Locusta*, the Gentian Violet chromosome technique stains the neck granule intensely violet. Such preparations have been shown to us by Mr. M. J. D. White of University College, London, and are at present being worked out.

of a row of neutral red stainable globules which have run together. No material is known to us which shows so well how different in morphology may be the Golgi apparatus of neighbouring cells. This fact is brought out in fig. 1, Pl. 1, where the Golgi apparatus may be filamentous as in the Sertoli cell, semi-dispersed as in the spermatogonia, and sub-spherical as in the older spermatocytes. The dictyokinesis is peculiar and rather surprising, and it is evident that the centrioles have little, if anything, to do with the assembly of the early spermatid Golgi bodies. The Golgi remnant appears to slough off without swelling up to form fat (2).

We have not included anything about the 'vacuome' and the 'active and inactive chondriome' in this paper. The reader who wishes to have our views on this topic as applied to mammalian spermatogenesis will find them in a recent paper by Gatenby and Duthie (10). We may say here, however, that the mitochondria are granular, not filamentous; there is no evidence as to their special degree of activity or inactivity except in so far as they definitely form the sheath of the middle-piece; they have no direct connexion with the Golgi apparatus, so far as we can ascertain; they can be stained a different colour from the latter by a number of methods; we know of no methods which stain both types of body the same shade of colour; both categories of cytoplasmic bodies are distinct in every sort of testicular cell, and we do not know whether the Y-granules which do stain in neutral red<sup>1</sup> are the homologues of the neutral red globules or so-called 'vacuome' in plants.

As we have mentioned, in no case are the mitochondria filamentous, but we have not found out anything new about them. The question of the post-nuclear region of the sperm head is interesting. Our material abounds with cells like that in fig. 26, Pl. 4, in which the early post-nuclear cap is so clear. No subsequent investigator will fail to find this cap in properly prepared Weigl or Kolatschew slides, and it is likely that by Da Fano or Cajal's methods the post-nuclear cap will be followed back to rudiments in the early spermatid. From stages fig. 9 to fig. 12, Pl. 2, we feel perfectly happy about our interpretation

<sup>1</sup> See footnote, p. 9.

of this membrane, but we cannot say whether it is ever a separate rudiment in the cytoplasm of the spermatid, or merely a thickening on the nuclear membrane.

On the question of the neck region we are not so clear. It is certainly incorrect to say that the centriole divides into three parts while it is still on view in the cytoplasm. The reader may be assured that our account up to fig. 12, Pl. 2, or in figs. 27, 28, and 26, Pl. 4, is correct. No critical observer could make a mistake in the class of material we have, but exactly what happens when the head centriole gets stuck on to the nucleus after stage fig. 34, Pl. 4, is unknown to us. It seems certain that the little projection shown in fig. 9, Pl. 2, at NBx. does not appear in our Benda preparations and is not centriolar in nature. But no one could say for sure that it is the rudiment of the neck apparatus, and we only think it is. It disappears between stages figs. 10 and 11, Pl. 2. It may be the body seen in some nearly ripe sperms as in fig. 29, x., Pl. 4.

Regarding the Y-granules (rubrophile granula), we have put them into the figure in Pl. 2, because we feel sure they are in the cell (9), but they cannot be seen for certain in osmic preparations, as in the cavy. However, there are several bodies usually to be seen which might be the Y-granules, but we do not know this for certain. The question of an accessory body enters here, and we both believe that one does exist in the human spermatocyte (fig. 4, ABx., Pl. 2) and probably some of the spermatids. The significance of these bodies is unknown. There is something of the sort in the cavy, and it divides regularly by itself at the prophases of mitosis (17).

The intercellular spaces are interesting. They are mainly parts of the Sertoli cell protoplasm, and the spermatogenetic cells lie much more in a 'nutrient syncytium' than is usually imagined.

In our figures in Pl. 2 we have adopted the interpretation of Painter and Evans and Swezy with regard to the presence of a Y-chromosome in the human. We have seen a number of mitoses which seem to support their views, but in the present paper we do not feel ready to say anything explicit in this matter.

## SUMMARY.

The points which we have brought out in this paper are:

1. The development of the acrosome from the Golgi apparatus, which has been figured for the first time during growth, and the stages of acrosome formation in the human.
2. The almost complete break-up of the Golgi apparatus at dictyokinesis, and the late reassembly of the fragments apparently independently of the spermatid centrioles.
3. The very probable presence of a neck granule apparatus as distinct from the head or proximal centriole ( $c^1$ ).
4. The claim that the head centriole does not divide. The emergence of the flagellum from the proximal and distal centrioles jointly.
5. The development of the post-nuclear cap in human spermatids.
6. The apparent absence of any form of spiral body in the middle-piece.
7. The different types of Golgi apparatus in the Sertoli and spermatogenic cells.
8. The presence of a vacuole in the head of the spermatozoon.
9. The remarkable 'nutrient syncytium' connected with the Sertoli cells.
10. The accessory body in the cytoplasm.

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## DESCRIPTION OF PLATES 1-4.

### PLATE I.

Fig. 1.—This is a small part of a cross-section of a spermatic tubule, whose fibrous wall is marked *fs.*, the cells above being interstitial, those below spermatic. On the left is a blood-vessel, the corpuscles of which are somewhat compressed; next are a number of interstitial cells containing the strikingly large Reinke crystalloids, as well as spherical granules. Below the fibrous wall (*fs.*) and adhering to it are two types of cells—on the left a Sertoli cell containing basally fat granules and a Charcot Böttcher crystalloid, and distally a long branched Golgi apparatus. The cytoplasm of these cells usually stretches inwards between the spermatocytes and to the lumen of the spermatic tubule, and here the heads of the ripening sperms are embedded. Just below the fibrous wall (*fs.*) is the second category of cells found here, namely the spermatogonia. These sometimes contain the baton-shaped crystalloid of Lubarsch. They have a semi-dispersed Golgi apparatus forming a close cap on one end of the nucleus. The cytoplasm of these cells is watery. Such cells undergo mitosis with forty-eight chromosomes and bud inwardly the third category of cells or spermatocytes, of which four are shown in different growth stages. In them the forty-eight maternal and paternal chromosomes pair, preparatory to the reducing or first spermatocyte division. During growth the Golgi granules shown in the spermatogonium run together to form the idiozome or Golgi apparatus, which is a large body applied to one side of the nucleus. Inside this granules are secreted which are the forerunners of the acrosome or head cap of the spermatozoon. The two spermatocyte divisions produce four spermatids (with the reduced number of twenty-four chromosomes) shown at the bottom right of the diagram. These spermatids metamorphose into spermatozoa, bottom left. In addition to the parts mentioned above, attention may be drawn to the forming acrosome in the spermatids in the bottom right, the acrosome bead having been deposited on the nuclear

membrane, whilst the two centrioles just at the edge of the cytoplasm have grown out a flagellum. In the spermatids at the bottom middle, the acrosome is now a clear cap in the front of the nucleus, and the post-nuclear cap has formed behind, the two caps or cups eventually meet and cover the head or nucleus, as on the left bottom where nearly ripe sperms are shown. In these most of the mitochondria have assembled on the middle-piece between the proximal and distal (ring) centrioles.

#### PLATE 2.

Figs. 2-13.—This is a slightly diagrammatic representation of the metamorphosis of the spermatozoa from the spermatogonia. Small examples of the two types of basal cells of the spermatid tubules are represented in fig. 2, the Sertoli cell on the left, containing fat granules and needle-shaped Charcot-Böttcher crystalloid, and a Golgi apparatus (sg.) distally. On the right is a spermatogonium, containing a baton-shaped Lubarsch crystalloid, Golgi elements (srg.), and mitochondria (m.). In fig. 3 the Golgi granules of the young spermatocyte have grown and have begun to run together to form a single large Golgi apparatus shown in fig. 4. In this figure the chromosomes in the nucleus are now arranged in pairs (tetrads) preparatory to their separation in the first or reducing division. The Y-granules which stain deeply in Nile blue and neutral red can be seen clearly at these stages. Inside the chromophobe part of the Golgi apparatus (idiozome, archoplasm) small granules appear within vacuoles, and these are apparently identical with the granules which form the acrosome or head cap of the ripe sperm, and are therefore called pro-acrosomic granules (PAG.). In fig. 4 the centrosome lies on the nuclear membrane between the nucleus and the Golgi apparatus, sometimes embedded in the latter. At the prophase of mitosis the Golgi apparatus shatters and becomes dissipated in the form of small granules in the cytoplasm, as shown in fig. 5, GA. These granules tend to keep near the spindle but not necessarily near the asters, especially during the last stages of mitosis, the arrangement in a telophase being shown in fig. 6. The mitochondria tend to lie towards the middle of the dividing-cell at this stage. After the two divisions four spermatids, of which fig. 7 is an example, are produced. In the newly formed spermatid the Golgi elements are somewhat scattered to one side of the cell and eventually assemble to form a single Golgi apparatus which contains a bead, fig. 8, which is deposited upon the nucleus to form the acrosome as depicted in fig. 9. This bead has a small vacuole around it and, after deposition as in fig. 9, the vacuole grows somewhat, forming a semi-lunar space (sr.) over the bead or acrosome. The Golgi apparatus soon detaches itself from the vacuole and flows down as a useless Golgi remnant (gr. in figs. 10, 11, 12, 13) finally discarded.

After the end of the second spermatocyte division in fig. 6 the centriole is left in each newly formed spermatid fairly near the cell-wall, up to which it passes, dividing into two (fig. 7,  $c^1$ ,  $c^2$ ), and jointly from them a flagellum



breaks the surface and grows out. As the cell grows older the double centriole moves inwards and can be seen now to consist of two different parts, the head proximal or first centriole, granular in nature and giving rise to the flagellum or axial filament, and the ring, distal or second centrosome which actually becomes a hollow ring threaded on the filament. The two sections adhere for a considerable time, moving in together and becoming applied to the side of the nucleus opposite to that on which the acrosome head has been attached, fig. 9. Subsequently the ring grows very considerably, becoming a most conspicuous object.

Now between the stages of fig. 9 and fig. 10 a new structure appears around the centrosomes and is applied to the nucleus. This is the post-nuclear apparatus or body which eventually covers the lower half of the ripe sperm head, the upper half being covered by acrosome (fig. 13, A., and PNC., Pl. 2). The acrosome head spreads rapidly during stages in figs. 10 and 11, and ultimately becomes thinner and less easily stained, as also does the material of the post-nuclear cap. When the nucleus of the spermatid has become ovoid and the two centrioles are still lying just behind, a sort of collar or manchette grows down from the nucleus and makes a small funnel as in fig. 10, MCH. This soon disappears and its exact significance is not known.

After fig. 11 the gap left by the down-growing acrosome cap and the up-growing post-nuclear cap becomes obliterated and the sperm head may be said to be formed completely, but the tail is not yet finished. Between the stages marked by figs. 11 and 12 the ring or second centriole ( $c^2$ ) slips down the axial filament a little way, actually a distance about the length of the sperm head, and stops, fig. 12; most of the mitochondria now crowd on to this space and fuse to form the mitochondrial middle-piece of the spermatozoon, MP., in fig. 13. The remnant of cytoplasm containing the remains of the cytoplasm, mitochondria, and Golgi apparatus is stripped off, as in fig. 13.

The ripe sperm, fig. 13, consists of a nucleus completely covered by two caps, the acrosome (A.) in front, the post-nuclear behind (PNC.), a head centriole ( $c^1$ ), a neck (NK.) or part between head centrosome and top of middle-piece (TM.), a middle-piece (MP.), and at the bottom of the middle-piece the shrunken ring centriole ( $c^2$ ). Beyond this is the flagellum (AF.) in two parts, a thicker and a lowermost thin tail.

It may be mentioned that a body (NBX.) appears at stage fig. 9 near the proximal and distal centrioles and persists for some time, eventually disappearing as such. It is thought to be a part of the neck granule apparatus and is not centriolar in origin or nature.

### PLATE 3.

Fig. 14.—Plan of human sperm according to Gatenby and Beams. The nucleus (stippled) is covered by two caps, a front one, the acrosome (A.), and a hind one, the post-nuclear cap (PNC.). The nucleus contains a

vacuole (v.). Into the base of the post-nuclear cap fits the neck (NK.) which contains a neck body (NB.), below which lies the head centriole ( $c^1$ ). The region between centriole 1 ( $c^1$ ) and centriole 2 ( $c^2$ ) is the middle-piece and is formed of a simple internal tube or skeleton (MPS.) upon which the mitochondria are situated.

Figs. 15-18.—Stages in the formation of the spermatozoon of the guinea-pig (*Cavia cobaya*) to illustrate the formation of the post-nuclear cap (PNC. in fig. 18) from post-nuclear rudiments or granules in fig. 15, PNG. The neck rudiment (NB.) becomes fragmented and finally forms a complicated structure connected to the undivided head centriole ( $c^1$ ). Prepared by Da Fano's method, and compiled from figures of Gatenby, Vejdovsky, and Meves.

Figs. 19-21.—Petrogale spermatids after Vejdovsky, showing independence of neck granule rudiment (RNB.) and centrioles ( $c^1$ ,  $c^2$ ).

Fig. 22.—Human spermatocyte stained supra-vitally in neutral red, and showing Golgi apparatus (GA.), accessory body (x.), Y-granules (y.), and crystals (z.). (After Gatenby.)

Figs. 23-5.—Robert Bowen's interpretation of human spermateleosis, drawn up by him after consulting previous authorities, PC. proximal and DC<sup>1</sup>, DC<sup>2</sup> distal centrioles.

#### PLATE 4.

All by Benda's stain (Alizarin and crystal violet).

Figs. 26, 30, 34.—Late spermatids.

Figs. 32, 27, 28.—Spermatids at earlier age, quoted in order of age of growth.

Fig. 29.—Nearly ripe sperm.

Fig. 31.—Spermatocyte.

Fig. 33.—Anaphase—telophase of dictyokinesis.

Note that figs. 26, 27, 28, 32, and 33 have been first treated by a Golgi apparatus osmic method and then stained in Benda. Figs. 30, 31, and 34 have been fixed in Champy and then stained in Benda.



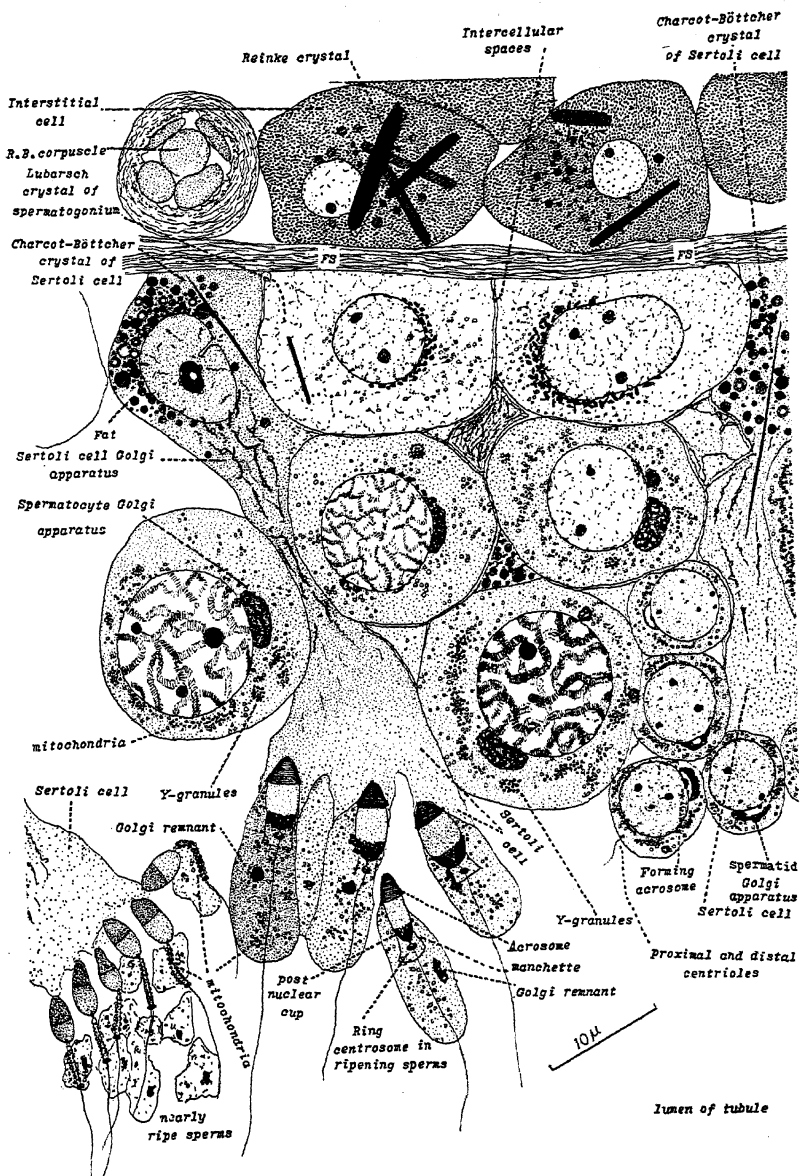
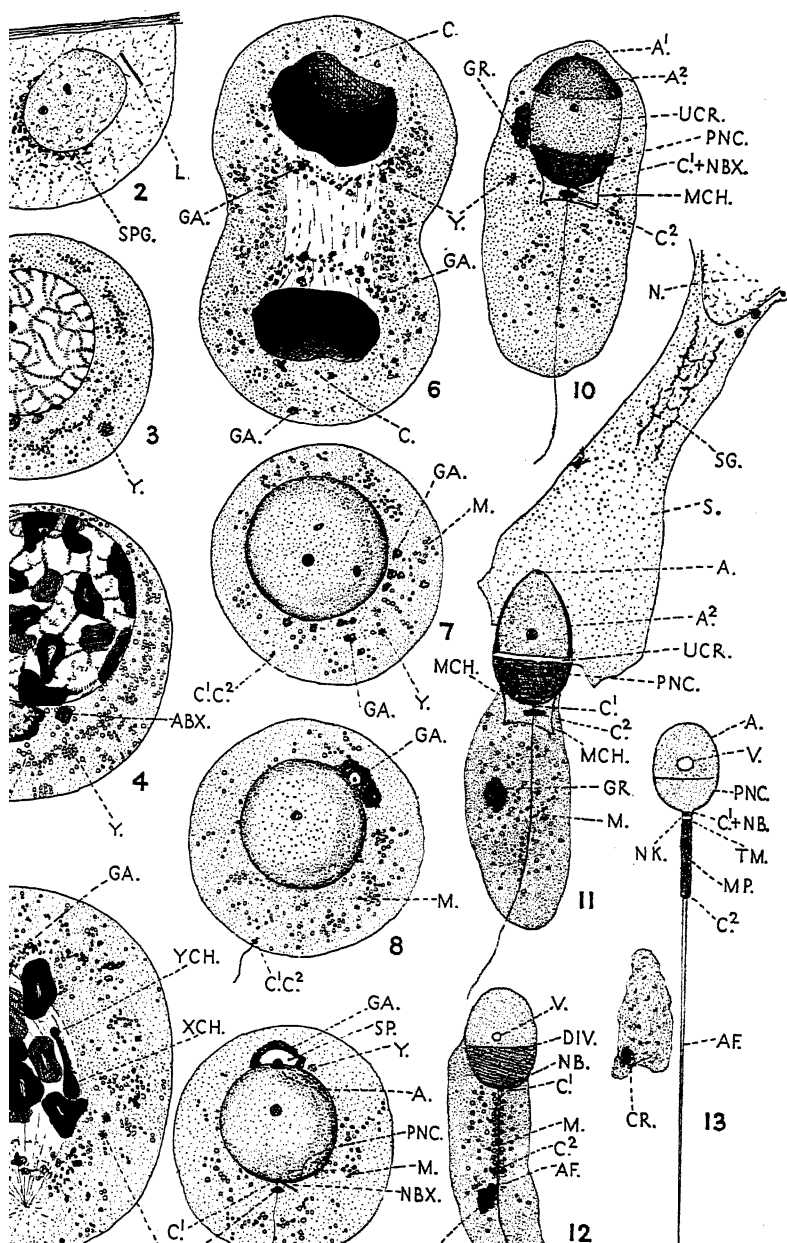
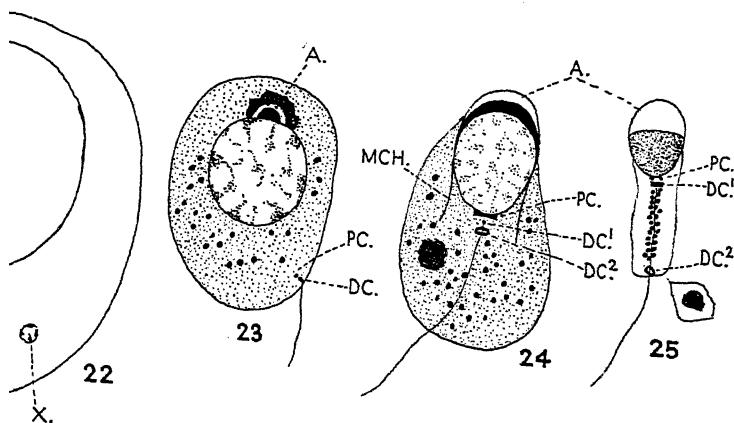
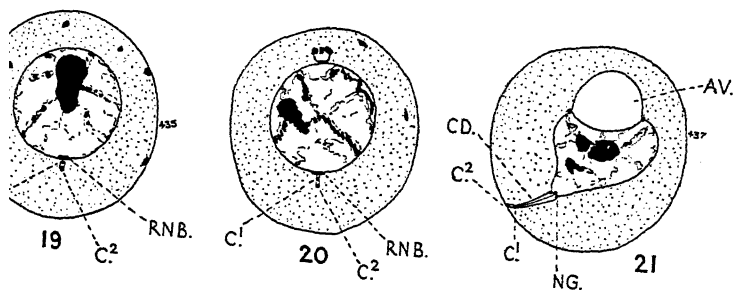
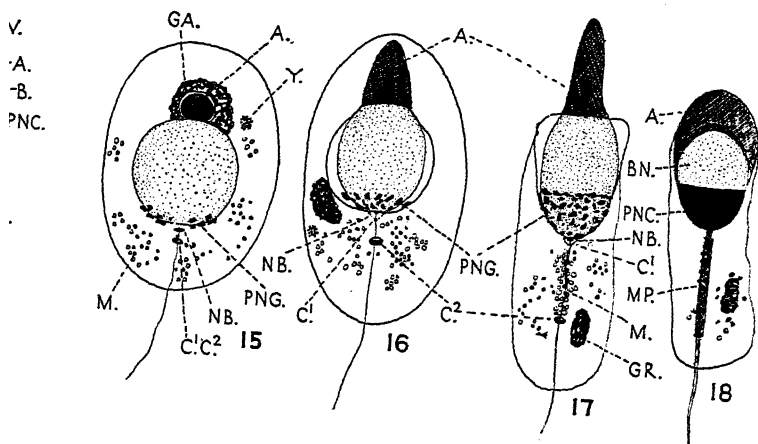


FIG. 1



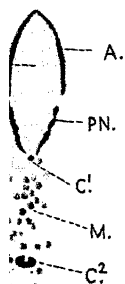






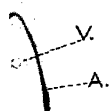






GA.  
AF.

26

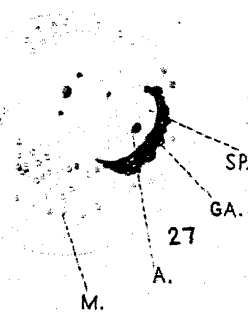


SP.  
C¹  
MCH.

C²  
GA.

30

AF.



27

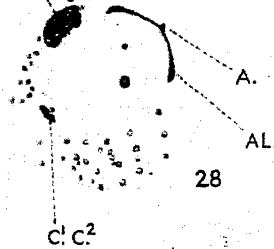
GA.

SP.

GA.

A.

M.



28

C¹ C²

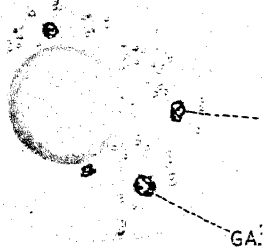
V.

X

C¹

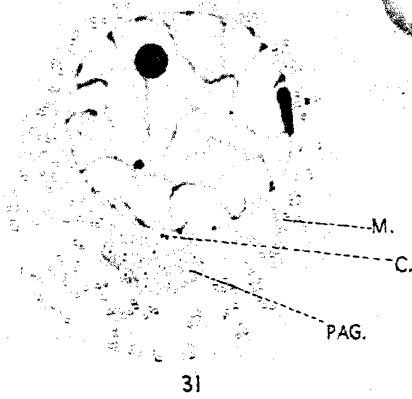
C²

AL.



32

GA.



31

M.

C.

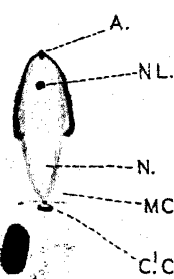
PAG.

CHR.

SPP.

GA.

M.



34

A.

NL.

N.

MC

C¹ C²

M.



# **The Revision, Systematic Position, and Origin of *Diplodinium* (*Polyplastron*) *multivesiculatum* and *Diplodinium* (*Polyplastron*) *bubali*.**

By

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With 7 Text-figures.

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*Diplodinium* (sub-genus *Polyplastron*) *multivesiculatum* (Dogiel and Fedorowa, 1925) undoubtedly is one of the most interesting representatives of the Infusorian family Ophryoscolecidae (which occurs in the stomachs of ruminants), especially from the systematic and phylogenetic standpoint. The origin of this species shows some peculiar causes which are operative in the development of these Infusorian species, but besides that the present treatise points out some new aspects regarding the development of new species in general.

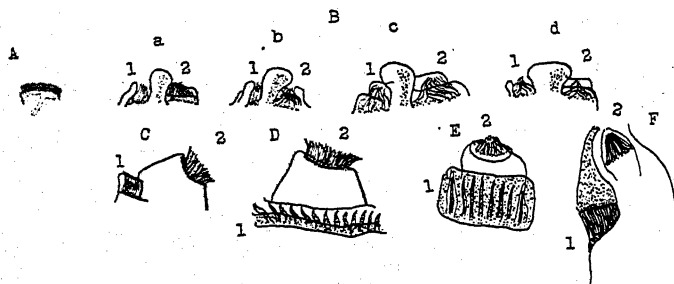
First of all the systematic position of the species *Polyplastron multivesiculatum* must be cleared up, because owing to some recent investigations it has become obscure and confused. This species was first discovered and described by Dogiel and Fedorowa in 1925 under the name *D. multivesiculatum*. In 1927 Dogiel established for this species a new sub-genus *Polyplastron*. In 1928 Dogiel described from the stomach of *Buffelus bubalus* L. another species of the same sub-genus, *D. (Polyplastron) bubali*. In their revision of the genus *Diplodinium* Schuberg, Kofoid and MacLennan have made *Polyplastron* an independent genus and have divided it into three species. The first species is the *P. multivesiculatum*, the second *P. fenestratum* (Dogiel, 1927) (this form was described by Dogiel only as an aberration of the species *P. multivesiculatum*), and the third is *P. monoscutum* (Dogiel's aberratio *confluens*).

For the species from the stomach of *B. bubalus* Kofoid and MacLennan have established a new genus with the single species *Elytroplastron bubali*. In 1933 Becker in a small treatise named the *E. hegneri* (Becker and Talbott, 1927) (with the following synonymy: *D. hegneri* Becker and Talbott, 1927, *D.-Polyplastron-bubali* Dogiel, 1928, *D.-Polyplastron-longitergum* Hsiung, 1931, and *E. bubali* Dogiel, 1928; Kofoid and MacLennan, 1932), which already in 1927 he had been the first to describe together with Talbott as a new species *D. hegneri*, from the stomach of domestic cattle and sheep. But as under this name both authors have described forms having a very heterogeneous structure Kofoid and MacLennan identified some of these forms described by the former authors (under the name *D. hegneri*) as *Ostracodinium obtusum* (Dogiel and Fedorowa, 1925). Becker agrees with them, but at the same time points out that some of the forms described by himself under the name *D. hegneri* can in no case be included in the group *O. obtusum* being already identical with the form described by Dogiel in 1928 as the new species *D. (Polyplastron) bubali*. Kofoid and MacLennan have discovered this form in the stomach of *Bos indicus*. In view of the law of priority according to the international rules of zoological nomenclature and of what has been said above, the name given by Becker and Talbott must alone be used although the latter's description was less complete than that of Dogiel, and although they have re-examined this form (Becker in 1933: 'The writer recently re-examined some of the type material of *D. hegneri*...') and only subsequently discovered (of course, on the basis of Dogiel's description) some characteristics formerly unnoticed by them and first described by Dogiel.

It is now most important to know whether Kofoid and MacLennan were right in raising *Polyplastron* from being a sub-genus of the genus *Diplodinium* to being an independent genus. They also made independent genera, of some other sub-genera, even of the species of the genus *Diplodinium*. In my revision of the family Ophryoscolecidae (which I am doing now) I shall, of course, touch upon every

single case; while in this treatise I shall only put forward such arguments as concern *Polyplastron*, making them, however, applicable in principle to all other cases. Regarding the seven genera of the family Ophryoscolecidae described up to the present day, *Entodinium* (Stein, 1858), *Diplodinium* (Schuberg, 1888), *Epidinium* (Crawley, 1924), *Ophryoscolex* (Stein, 1858), *Caloscolex* (Dogiel, 1926), *Opisthotrichum* (Buisson, 1923), and *Cunhaia* (Hasselmann, 1918), there exist, fortunately, among them such differences that no one can any longer confuse them. Besides, it must be emphasized that all representatives of the genera in question are as a rule so uniform and typical that it is easy to identify them. The chief generic difference is the structure of the ciliary or membranelle zone located in the anterior end of the body near to the mouth. This zone has a structure so uniform and characteristic in all representatives of the same genus that at the first glance through the microscope we may immediately determine the genus. Thus the genus *Diplodinium* is characterized by two membranelle zones (adoral and dorsal), both of them in the same transverse plane, while between the two zones is found a well-developed *processus apicalis*, whereby the genus *Diplodinium* is undoubtedly distinguished from other genera of this family (see Text-fig. 1). The genus *Diplodinium* was divided by Dogiel into four subgenera (*Anoplodinium*, *Eudiplodinium*, *Ostracodinium*, and *Polyplastron*), while Kofoid and MacLennan have raised all these subgenera into genera proper, adding also some new ones, so that according to them all that formerly was included in the genus *Diplodinium* is now separated into the following distinct genera: *Eodinium*, *Diplodinium*, *Eremoplastron*, *Eudiplodinium*, *Diploplastron*, *Metadinium*, *Polyplastron*, *Elytroplastron*, *Enoploplastron*, and *Ostracodinium*. They made this division by taking the more special characteristics (for example, the skeletal plates) as a criterion for the generic differentiation, which is a great logical error (*divisio*

sit adaequata!). In view of the fact that Ophryoscolecidae have several genera, some with skeletal plates and some without, the skeletal plates cannot properly be taken as a typical criterion for generic division (this would only be possible if the family had only two genera, of which one had skeletal plates and the other not). That this is really so may clearly be seen in the case of Ophryoscolex and Epidinium, because these two genera, so different and so sharply divided, have an identical structure

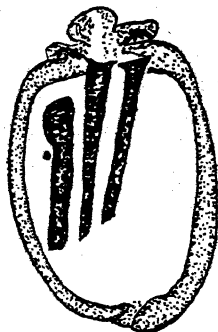


TEXT-FIG. 1.

Adoral and dorsal membranelle zones of the Ophryoscolecidae.  $\times 200$ . A, *Entodinium* sp.; B, *Diplodinium* sp.: a, *Anoplodinium* sp., b, *Eudiplodinium* sp., c, *Polyplastron* sp., d, *Ostracodinium* sp.; C, *Epidinium* sp.; D, *Ophryoscolex* sp.; E, *Caloscolex* sp.; F, *Opisthotrichum* sp.: 1, dorsal, 2, adoral membranelle zone.

of the skeletal plates. For this reason it is imperative to return to Dogiel's original division, as Kofoid and MacLennan's new genera, arrived at by the splitting up of the genus *Diplodinium*, are nevertheless more closely related to each other than to other genera of the family Ophryoscolecidae. Kofoid and MacLennan's division has two fundamental errors. The first one is of a theoretical nature. According to their division we make a mistake from the phylogenetic point of view, because all representatives of the phylogenetically uniform genus *Diplodinium* are being split into independent systematic categories taxonomically equivalent to other genera of the same family which are not so closely related to this group, while on the contrary genera should be

equivalent units. The second error is of a practical nature. Related groups are being split, and an entirely unnecessary ballast of new names is created, causing taxonomical and phylogenetical confusion. For this reason it is better to leave all the representatives of this group in one single genus *Diplodinium*. In so far as there is a necessity for further



TEXT-FIG. 2.

*P. multivesiculatum*.  $\times 300$ .  
Dorso-ventral right view.



TEXT-FIG. 3.

*P. hegneri*.  $\times 300$ .  
Dorso-ventral right view.

differentiation, *Diplodinium* may be divided into subgenera. According to the arguments stated above, and basing ourselves on the facts relative to the systematics of the family Ophryoscolecidae, we are actually forced to make a revision in the sense that *Diplodinium sensu latiore* be re-erected as a genus *sensu latiore*, with subgenera. One of the subgenera of the genus *Diplodinium* is *Polyplastron*, which interests us most here.

After this revision *Polyplastron* (see Text-figs. 2 and 3) has the following position:

Genus *Diplodinium* Schuberg 1888.

Sub-genus *Polyplastron* Dogiel 1927.

Species *P. multivesiculatum* Dogiel and Fedorowa, 1925.

*P. hegneri* Becker and Talbott 1927.

The following table shows the hosts of the two species as well as their synonymy.



TABLE 1.

*P. multivesiculatum* and *P. hegneri*: Investigators, hosts, and synonymy.

<i>Species.</i>	<i>Host.</i>	<i>Investigator.</i>
1. <i>P. multivesiculatum</i> . Dogiel and Fedorowa, 'Arch. f. Protistenk', vol. 59, p. 130, figs. 73, 74, 1927.  Synonymy: <i>D. multivesiculatum</i> . Dogiel and Fedorowa, 'Zool. Anz.', vol. 62, p. 100, fig. 4, 1925.	<i>Bos taurus</i> , <i>Ovis aries</i> , <i>Capra hircus</i> , <i>O. orientalis</i> <i>cycloceros</i> , <i>C. aegagrus</i> .	Dogiel and Fedorowa, Becker and Talbott, Wertheim.
2. <i>P. hegneri</i> . Becker and Talbott, 1927.  Synonymy: <i>D. hegneri</i> . Becker and Talbott (partim), 'Iowa St. Coll. Journ. Sci.', vol. 4, p. 357, pl. 2, fig. 17, 1927.  <i>D. (Polyplastron) bu-</i> <i>bali</i> . Dogiel, 'Ann. Para- <i>sitol.</i> ', vol. 6, p. 332, fig. 4, 1928.  <i>E. bubali</i> . Kofoid and MacLennan, 'Univ. Calif. Publ. Zool.', vol. 37, p. 121, pl. 6, figs. 13, 14, 1932.	<i>B. taurus</i> , <i>O. aries</i> , <i>B. bubalus</i> , <i>B. indicus</i> .	Becker and Talbott, Dogiel, Kofoid and MacLennan.

Before proceeding farther, we must examine why the erection of the species *P. hegneri* to the new genus *Elytroplastron* is unjustified and why we are justified, both phylogenetically and taxonomically, in including it in the sub-genus *Polyplastron*, which after all had already been done by Dogiel. Similarly, we must prove the fallacy of Kofoid and MacLennan's procedure when making Dogiel's aberrations of the species *P. multivesiculatum*, i.e. *P. multivesiculatum aberratio confluens* and *P. multivesiculatum aberratio fenestratum*, independent species. Dogiel, to whom we are indebted for a very complete description of

*P. hegneri*, points out that this species in general reminds one of the typical species *P. multivesiculatum* (Dogiel, 1928: '... très semblable au *P. multivesiculatum*'), having, however, distinct features of a separate species ('... font de *P. bubali* une bonne espèce'). Kofoid and MacLennan, too, when stating that 'although the paths of development of the two have been different', say, however, in regard to *P. hegneri* and *P. multivesiculatum*: 'Polyplastron exhibits approximately the same grade of complexity as Elytroplastron.' *P. hegneri* and *P. multivesiculatum* have the same general shape, the same structure of the ecto- and endoplasm (which is very important!), the same form of the macro-nucleus and micro-nucleus and of the two right skeletal plates; further they have the same shape and position of the rectum and of the anus. The only difference is in the number of accessory contractile vacuoles and in the absence of one of the left skeletal plates. These differences, however, are too small to justify the making of a separate genus. Kofoid and MacLennan themselves, for instance, have left the species *Metadinium medium* and *M. ypsilon* in the same genus *Metadinium*, although the first of these two species shows the two right skeletal plates clearly separated, while in *M. ypsilon* these two plates are fused (not to mention other notable differences such as the general shape and size of the body, the shape and position of macro-nucleus and micro-nucleus), and again Kofoid and MacLennan say themselves (1932, p. 60): 'The skeletal plates are very constant, highly characteristic structures,' and further (1932, p. 118): 'In the other genera, the form of the plates is stable within a species and it seems unlikely that Polyplastron alone would be an exception.' It is evident that, if they had been consistent, they would, in view of the above-mentioned example, also have to divide the genus *Metadinium* into new and separate genera. If, in view of the great variability of all characteristics including the skeletal plates of the family Ophryoscolecidae, their method were adopted, almost every second species would make a separate genus. Furthermore, the same authors say again (1930, p. 497): 'The most marked differences are presented

by the macro-nucleus, both in position and shape, position of the contractile vacuole, the endoplasmic sac and rectum, and finally the external characters such as shape, size,<sup>1</sup> and the various projections in the form of lobes, spines, and flanges. No one character is sufficient to completely characterize a species and to establish its relationships within the genus. The whole complex of characters must be used.' Accordingly, both authors contradict themselves not only in the facts but also in their own theses when in one case they put individuals with different skeletal plates (besides other differences) into the same genus (the case of *Metadinium*), while in another case they split, for the same reasons, two species into two separate genera (the case of *Polyplastron* and *Elytroplastron*), which they should never have done, if they had perceived not only the morphological similarity but also the phylogenetic origin of the two species in question (Kofoid and MacLennan, 1932, p. 121: 'It may be suggested that *Elytroplastron* and *Polyplastron* have evolved from a form such as *Diploplastron* and in the same direction.') On the ground of the above-stated arguments it is incorrect to divide these two species into two separate genera, a division into two clearly characterized different species of the same genus *Diplo-dinium*, sub-genus *Polyplastron*, being quite sufficient.

In addition to the typical *P. multivesiculatum*, Dogiel has described two more aberrations, i.e. *confluens* and *fenestratum*, the first one with totally fused right skeletal plates, the second with partly fused skeletal plates. Kofoid and MacLennan have erected these aberrations into separate species: *P. monoscutum* (instead of using Dogiel's name *confluens*, here Kofoid and MacLennan have quite correctly acted in conformity with the Art. 11 of international rules regarding zoological nomenclature, the name *confluens* having already been occupied) and *P. fenestratum*. Dogiel has rightly named these forms 'aberrations', because he seldom found them, in great contrast with the very frequent

<sup>1</sup> The identity of the characteristics enumerated here relating the *P. multivesiculatum* and *P. hegneri* having already been mentioned before.

appearance of the typical species *P. multivesiculatum*. The aberration confluens Dogiel saw only once! In fact, it is impossible to erect a new species on a single aberrative individual! I had an opportunity myself of examining during a year very rich populations of *P. multivesiculatum* of Somali sheep, having used every day fresh material from her stomach, and observed them both living and fixed (stained). During several months the *P. multivesiculatum*, by the number of individuals, was the predominant member of this



TEXT-FIG. 4.

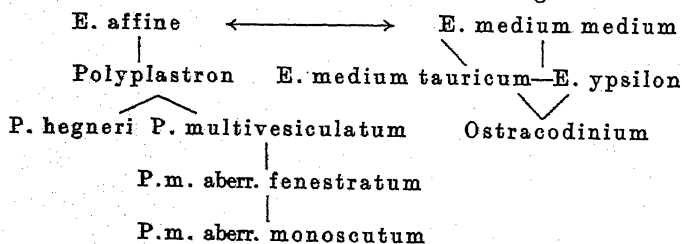
Transitional stages of the fusion of the right-side skeletal plates of *P. multivesiculatum*.  $\times 300$ .

stomach infusorian fauna. The great number of individuals gave me a wide choice. By careful observation of the forms and the position of the two right skeletal plates (primitiva and carina) in more than 700 individuals belonging to this species, I could see myself that in rare cases the gradual fusion of these two skeletal plates was to be observed. Such fusion shows all possible transitional stages (see Text-fig. 4).<sup>1</sup> In view of the great scarcity of such forms, we cannot speak in this case of any separate form, much less of a separate species; the more so as they differ but slightly from the type, and as there exists a series of gradual transitional stages which show that this form is a simple, individual, aberrative variation. Accordingly, these so-called 'species' *P. monoscutum* and *P. fenestratum* must be discarded! Just these aberrant forms reveal to us the direction in the evolution of the family Ophryoscolecidae and its great variability. Dogiel has shown (1925) how this variability

<sup>1</sup> By using chlor-zinc-iodide or a combination iodine-alcohol and concentrated  $H_2SO_4$  one can get a nice reaction (brown to black) of the skeletal plates.

is sometimes also conditioned and considerably increased by a special formation of some internal anatomical characters.

Now it is necessary to examine the position of the sub-genus *Polyplastron* within the genus *Diplodinium* and to find the line of evolution of the two very significant species *P. multivesiculatum* and *P. hegneri*. The complex structure of the skeleton and the vacuolary apparatus of these species must be deduced from one of simpler structure. If all these characters are taken into consideration, there is only one form *D. (Eudiplodinium) affine* (Dogiel and Fedorowa, 1925) (synon.: *D. affine* Dogiel and Fedorowa, 1925, after Kofoid and MacLennan, 1932) which is the nearest to *Polyplastron*. In this I fully agree with Kofoid and MacLennan, who (as cited above) very correctly noted the relation between these species. From one single, narrow, skeletal plate of the same species of the *Diplodinium*—sub-genus *Eudiplodinium*—leads the way to those species of *Eudiplodinium* which are equipped with two relatively narrow, right-side, skeletal plates, and these are *E. affine* and *E. medium*. However, in the case *D. (Eudiplodinium) medium tauricum* these two skeletal plates that extend from the posterior part fuse into a single one (with transitional stages), leaving anteriorly a greater or smaller opening. We are, therefore, right in assuming that the wide, right-side, skeletal plate of the *Diplodinium*—sub-genus *Ostracodinium* (Dogiel, 1927)—has evolved from two narrower ones which had gradually fused into a single one. Dogiel, too, confirms this opinion. In this way *E. affine* would be a progenitor of *Polyplastron* and *E. medium* of the *Ostracodinium*, as we see in the following scheme:



It follows therefrom that *Polyplastron*'s position

between *E. affine* and the descendants of *E. medium* medium, being, however, itself distinguished from the latter as a separate group with orimantal development of left-side skeleton preserving, but slightly, their tendency for the fusion of *primitiva* and *carina*. Parallel with this growth of the skeletal plate goes the increase in number of contractile vacuoles, as will be shown in the following table:

TABLE 2.

Enlargement of the skeletal plate and increase in number of contractile vacuoles in the developmental line.

Eudiplodinium { Polyplastron  
Ostracodinium

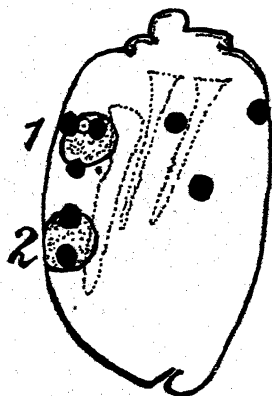
<i>Species.</i>	<i>Skeletal plates (right).</i>	<i>Number of contractile vacuoles.</i>	<i>Notice.</i>
<i>E. affine</i>	2 of middle width	2	If these were not a large right-side skeletal plate, there would be no obstacle for a similar development, in addition to a number of vacuoles along the dorsal border, of another 'irregular' vacuole, which can be assumed by the fact that with the <i>P. multivesiculatum</i> aberr. <i>monoscutum</i> the 'irregular' vacuole No. 7 disappears, because it was 'driven out' by the fused skeletal plate.
<i>E. medium medium</i>	2 of middle width	2	
<i>E. medium tauricum</i>	are fusing	2	
<i>P. hegneri</i>	2 right of middle width	3 or 4	
<i>P. multives.</i>	2 right of middle width <sup>1</sup>	9	
<i>O. obtusum obtusum</i>	1 large	6	

According to the report of Kofoid and MacLennan and Talbott, *P. hegneri* was found by them also in domestic cattle; thereby extending our knowledge regarding its occurrence. The only host known before was *B. bubalus*, according

<sup>1</sup> With a tendency for fusion.

to Dogiel. According to their structure, *P. multivesiculatum* and *P. hegneri* belong to the most complex descendants of the family Ophryoscolecidae. They belong to the most evolved forms. From Eudiplodinium, having a constant number of two vacuoles, Polyplastron emancipates itself with a tendency to increase this number. However, it is still a question why this number is increasing. My experimental investigations of a number of vacuoles have led me to realize the fact, which I could also ascertain numerically (for the *P. multivesiculatum* too), that the total surface and the size of the contractile vacuoles stand in a definite relation to the total surface and the size (Wertheim, 1934) of a certain species of the family Ophryoscolecidae. If we compare the size of the contractile vacuoles of *P. multivesiculatum* with the size of those of *D. (Eudiplodinium) medium*, for example, we see how small are those of *P. multivesiculatum*, and their number ought therefore to be proportionally greater in order to enable them duly to carry on their task. We therefore can establish the rule, that in the family Ophryoscolecidae the number of contractile vacuoles increases only parallel to the increase of that given species, or as well by a simultaneous decrease in the size of some existing vacuoles. This can be clearly observed in the case of the genus Ophryoscolex as well as with the species *O. obtusum* (Dogiel and Fedorowa, 1925). While studying the already mentioned abundant populations of *P. multivesiculatum*, I could ascertain that there were a few atavistic cases in which the forms *P. multivesiculatum* did not have the usual nine vacuoles but only two dorsal ones; but those were now much larger (in accordance with our above-mentioned rule; see Text-fig. 5.) These vacuoles, owing to their size, were influential in changing the form of the macro-nucleus. This is an analogous case—in a different direction—to that of Dogiel's (1925). The case of atavistic recurrence in these two vacuoles, typical for Eudiplodinium, is another proof of the fact that Polyplastron was derived from Eudiplodinium. Concerning the skeletal plates, I could ascertain a gradual transition to the

Eudiplodinium type with two right-side skeletal plates. Text-fig. 6 shows the orimental development of the left-side



TEXT-FIG. 5.

Contractile vacuoles of *P. multivesiculatum*.  $\times 400$ .

skeletal plates as I could ascertain them in the populations of *P. multivesiculatum* from the Somali sheep mentioned above. Evidently, this enlargement of the skeletal apparatus



TEXT-FIG. 6.

Oriments of left-side skeletal plates of *P. multivesiculatum*.  $\times 400$ .

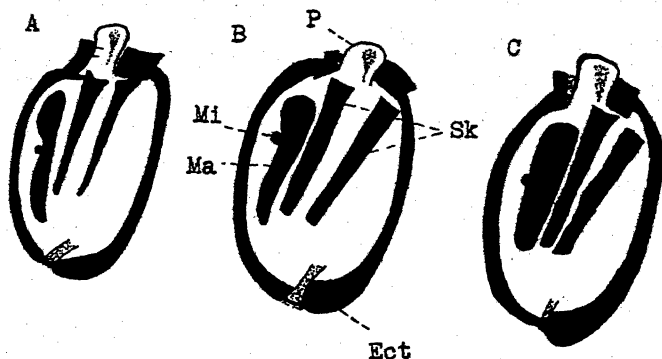
means intensification of its function. Using chlor-zinc-iodide as a reaction for a stronger brown to black staining of the skeletal plates (reaction on the cellulose) I could observe that with the forms without skeletal plate a collapse would take place at a great concentration of the reagent, while with the forms having right-side skeletal plates it was somewhat slighter, as well as with *Ostracodinium*, while *Epidinium* and *Ophryoscolex* were almost perfectly resistant. This means that the



latter are protected by the skeleton, which in this case envelops their body all around. Consequently, regarding the function of these skeletal plates, I can, on the basis of my observations, confirm the following fact: that the skeletal plates have also the function of strengthening the bodies of these forms, not alone of the pharynx. I suppose, owing to a very dense stomach-liquid, the body of the Ophryoscolecidae must be resistant against lateral pressure from all sides. The cuticle, strengthened by a thick layer of ectoplasm (encrusted by silicic acid), has to serve this purpose, but in any case the skeletal plate may intensify this action. It is characteristic that the relative thickness, of course, with regard to the size of the species in question, of the ectoplasm is less as the skeletal apparatus is more developed. To support my assertion I also mention the fact that the skeletal plates usually develop on the right and the left side of these Infusoria (not dorso-ventrally), but as their body is dorso-ventrally flattened they are larger on the right and left sides. Where the surface is larger there is also a greater pressure, so that consequently a greater resistance and hardening of the sides of the body are necessary. According to this, cylindrical forms again (Epidinium, Ophryoscolex) have not a pronounced right-side skeletal apparatus but a cylindrical one, because while in movement their more rounded body is more evenly exposed to pressure of the environmental fluids. The same applies to Caloscolex and Opisthotrichum. Ostracodinium is in this respect intermediate. If we consider these two representatives of the sub-genus Polyplastron, we shall observe that *P. multivesiculatum* shows greater complexity than *E. affine* (compare in Text-fig. 7 the identical parts in the bodies of *P. multivesiculatum*, *P. hegneri*, and *E. affine*). In all probability the form *P. hegneri* is phylogenetically older. As we can see from our comparative drawings, *P. hegneri* is closer to *E. affine* in the position of its dorsal vacuoles, by the form of its macro-nucleus, and by the direction of the right skeletal plates, than is *P. multivesiculatum* to the *E. affine*. Thus a cue is given for the tendency of the development of left-side skeletal plates, as

shown in the present case of *Diplodinium*—sub-genus *Polyplastron*.

Finally the ways in the evolution of the *P. multivesiculatum* and *P. hegneri* must be explained, these two species being very interesting by the tendencies in their evolution, and as having a distinctive structure of their skeletons and their contractile vacuoles. These species show how the evolution of *Ophryoscolecidae* opens up very many possibilities in a most



TEXT-FIG. 7.

A, *P. multivesiculatum*, B, *P. hegneri*, C, *Eudiplodinium* affine (comparative drawings, dorso-ventral right view). P., processus apicalis; Ma., macro-nucleus; Mi., micro-nucleus; Sk., skeletal plates; Ect., ectoplasm. A,  $\times 225$ ; B,  $\times 300$ ; C,  $\times 750$ .

peculiar way. And the two species in question, which in the genus *Diplodinium* have their own place, have just realized one of those possibilities. They represent a new evolutionary type which in the future can in this direction produce quite independent groups, the more so as I already proved (Wertheim, 1934, Zool. Anzeiger) a 'caudopetal' tendency in *P. multivesiculatum*. Speaking of the factors at work in the evolution of *Ophryoscolecidae*, it must first of all be pointed out that here the factor of selection in the form of some struggle for life can in no case come into consideration, which is self-evident.

There is only one thing that must be kept in mind: great

masses of these forms perish daily with the passing of the food into the omasus and abomasus, where actually their remains may be found. As my former investigations have shown, the species of Infusoria are fairly uniformly distributed in the stomach so that representatives of all species always remain living in a given stomach. Therefore this 'incidental selection' would not be decisive. The factor of adaptation to the dense environment is of greater importance, which adaptation in turn favours the development of the skeleton and the caudal projections, and these belong to the most characteristic criteria for the differentiation of the Ophryoscolecidae. Therefore the adaptation here should be regarded as a motive factor in their evolution. Since these Infusoria, however, live in the greatest variety of different environments, i.e. in the greatest number of ruminants' stomachs, the oecologic factors, too, must be considered. For instance, the same conditions never exist in all those stomachs; the more they differ (on account of different food or some purely physiological causes), the greater is the possibility of differentiation. However, this oecologic factor is a regulative one, not motive, as in all these various stomachs the adaptation as a reaction to all given special circumstances is of primary importance. As far as we know to-day, Ophryoscolecidae are transmitted through contact per os from ruminant to ruminant. This would be in conformity with the already established fact that those groups of ruminants where this contact for any reason was possible in the past but is no longer possible in the present, show a different structure of the species in their infusorial fauna. This is the factor of isolation. It, too, is a regulative factor. The isolation in a narrower sense (in the same locality, mechanical, if on account of difference in size no contact per os takes place; in a small Cameroon goat and a camel, for example, kept in a zoological garden) as well as through geographic isolation may cause a diversity in the several local faunas, simply because infections and reinfections take place through contact per os. Here, in other words, we come to the conclusion that all factors in the evolution are not equivalent at all, that they do not lie in the same dimension, on the same level.

Regarding *Polyplastron* and its 'caudopetality', the structure of the skeleton and of the vacuolar apparatus is a result of the motive factor of adaptation; while the fact that in certain environments some of its varieties are more isolated is a result of oecologic difference or of geographical isolation acting as regulative factors. Thus we can see clearly why *P. multivesiculatum* was found to be predominant in Europe, the United States of America (primarily), and Northern Persia, while in Eastern Asia, in tropic regions, and in the United States of America<sup>1</sup> *P. hegneri* is predominant. Besides, *P. multivesiculatum* is a distinctive inhabitant of the stomach of domestic cattle, sheep, and goats, while *P. hegneri* is characteristic (besides being present in domestic cattle and sheep) for *B. bubalus*. Thus the evolution of a certain species depends not only on its particular but also on its other properties, and consequently on the evolution of the species as a whole. And the evolution of the species as a whole is a consequence not of any isolated evolutionary factor, but of a number of factors which are interdependent and which act as a unity as cogs in cogwheels. There are great chances of applying successfully to other forms this method of analysing the evolution of species derived from the study of these Infusoria, rightly held by all investigators to be of exceptional interest. For example, Becker observes that they are 'an assemblage of unicellular organisms unexcelled in complexity and diversity by any other ecologic groups' (1932, p. 282). Owing to the great importance of these Infusoria it is indispensable to first put in order their systematics as a basis of all further cytological, biological, and physiological investigations.

#### SUMMARY.

In revising the species of the *Diplodinium* (sub-genus *Polyplastron*) the author comes to the conclusion that on the basis of the generic criteria in the family Ophryoscolecidae, two distinct genera cannot be erected as was done by Kofoid and MacLennan (the genera *Polyplastron* and *Elytro-*

<sup>1</sup> In the United States of America *P. hegneri* is a secondary immigrant (of *Bos taurus*).

plastron). On the contrary it is justifiable to include both of these species in Polyplastron (Dogiel, 1927) as a sub-genus of the genus Diplodinium. In conformity with the international rules of zoological nomenclature, the correct names of both species in question are as follows: *D. (Polyplastron) multivesiculatum* Dogiel and Fedorowa, 1925, *D. (Polyplastron) hegneri* Becker and Talbott, 1927. Evidence is put forward to show how incorrect is the erection of Dogiel's aberrations *P. multivesiculatum* 'confluens' (monoscutum) and *P. multivesiculatum* 'fenestratum' into separate species, and how these forms are to be considered only as individual varieties. These views are supported by the author's own investigations on live and fixed (stained) material.

The author agrees with Kofoid and MacLennan that Polyplastron had evolved from forms related to *D. (Eudiplodinium)* affine, and brings forward arguments resulting from his investigations as to how this relation can be clearly disclosed. He also gives a scheme of the systematic position and phylogenetical relationship of the species in question of Diplodinium—sub-genus Polyplastron.

Conclusions are drawn concerning the relation and kinship of *P. multivesiculatum* and *P. hegneri*, as well as the author's rules in regard to the development of new contractile vacuoles, also the reasons for the development of the skeleton and the position and form of skeletal plates. These skeletal plates serve for a general strengthening of the body besides other functions.

It is argued that adaptation is a motive factor in the evolution (the selection appears as an 'incidental selection'), while the oecologic factors and the factors of the mechanical and geographical isolation of the ruminant-hosts (in harmony with the per-os manner of infection of the ruminants by these Infusoria) act as regulative factors. These two categories of factors are in diverse dimensions (they are non-equivalent and inadequate). The evolution of characters cannot be considered singly, but only species as units, and this evolution is not a result of isolated

factors but of a series of factors permanently acting in reciprocal dependence. The author considers this method of analysis applicable also to other systematic groups.

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# The Histology, Cytology, and Embryology of Sponges.

By

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THE classical investigations which form the foundation of our knowledge of the minute structure of sponges were mainly carried out in the latter half of the nineteenth century by Haeckel, Schulze, Sollas, Poléjaeff, Topsent, Minchin, Maas, and other workers. In the earlier years of this century while many papers were published dealing with taxonomy, skeletal structure, physiology and descriptions of new species, the study of the soft parts was somewhat neglected, though the work of Minchin forms an important exception. More recently, however, a revival of interest in these aspects, assisted by modern fixing and staining methods, has resulted in a considerable increase in our knowledge. It is the aim of this paper to give a résumé of the literature published during the last twenty year which deals with those aspects of sponges indicated in the title. 1914 has been chosen as the beginning of the period since all previous references are available in Vosmaer's bibliography.<sup>1</sup>

## HISTOLOGY.

The most recent, and perhaps the most complete histological study is that of *Reniera elegans* (Bwk.) and *Reniera simulans* (Johnst.), two well-known members of the Haploscleridae, by Tuzet (1932). She describes the following types of cell:

(1) *Exopinacocytes*, which form an epithelium covering the entire outer surface of the sponge except where it is pierced by pores. (Strictly speaking, according to the terminology employed by Minchin the word 'pore' is applicable to the superficial orifices only in Ascons. In all higher types the superficial

<sup>1</sup> Vosmaer, G. C. J., 'A Bibliography of Sponges, 1551-1913'. Cambridge, 1928.



orifice is an ostium, while the homologue of the pore is the 'chamber-pore' or prosopyle. The word 'pore' is, however, so widely used by French and American authors to denote ostia that one must be content merely to call attention to the ambiguity.) The pores are holes between these cells and not within a single cell. The exopinacocytes are flattened cells about  $14 \times 4\mu$ , and on their inner surface are produced into blunt pseudopodia which project into the 'mesenchyme' layer. The granular nucleus has no nucleolus.

(2) Endopinacocytes, which line all the inhalant and exhalant canals, i.e. form all the gastral layer with the exception of the lining of the flagellated chambers. They are similar to the exopinacocytes except that they are about half their length, possess no pseudopodia, and the nucleus contains a nucleolus from which radiate bands of chromatin.

(3) Choanocytes, of typical structure, confined to small spherical flagellated chambers. Each consists of a body,  $5 \times 3\mu$ , collar and flagellum. The nucleus is at the base of the cell and contains a nucleolus. The structures at the base of the flagellum will be discussed in connexion with cytoplasmic inclusions.

(4) Silicoblasts, which are elongated cells closely applied to the spicules. No cases were found of a spicule being included in a single cell, and the general consensus of opinion now is that the growth of the spicule is almost entirely extracellular. The silicoblasts have a reticulate nucleus with a nucleolus. These and all the following types are confined to the skeletogenous layer or 'mesenchyme'.

(5) Stellate cells, consisting of a round body about  $10\mu$  in diameter from which arise long fine pseudopodia which freely anastomose with the pseudopodia of other stellate cells and of exopinacocytes. The nucleus is granular, containing a nucleolus, and the cytoplasm has a finely granular structure in contradistinction to the previous types in which it is more or less hyaline.

(6) Amoebocytes, including the archaeocytes and phagocytes of many authors. They are amoeboid cells with many inclusions which presumably have been ingested. Their nucleus resembles that of the silicoblasts.

(7) Granular cells, which are of two kinds: (i) Those

with small, very numerous granules, which stain with silver, osmium, haematoxylin and fuchsin, but not with eosin, and do not stain blue with iodine. It is conjectured that they are lipoproteid. (ii) Those with larger granules (about  $1\mu$  in diameter). These granules stain with eosin after fixation in Bouin's fluid. In other respects the two types of cell are similar. In both there is a reticulate and nucleolate nucleus.

(8) *Globoferous* cells, which are characterized by a single large albuminoid inclusion that stains with fuchsin.

(9) *Fibroblasts*, the cells that secrete the spongin fibres, which in *Reniera* form a considerable part of the skeleton. In their early stages they resemble amoebocytes; during spongin formation they become vacuolated and eventually disintegrate, liberating the fibre. In *Reniera elegans* several cells become arranged in a row to form a single large fibre: in *Reniera simulans* each cell forms a small fibre independently. This distinction is interesting in view of the fact that Burton (1926), who has been attempting to restore order into the hopelessly confused taxonomy of the Haploscleridae, has decided to include both these as well as several others of Bowerbank's species within the single species *Reniera cinerea* (Bwk.).

Another full histological account, in this case of *Microciona prolifera*, has been given by Wilson and Penney (1930). Their description is based on the study both of sections and of the living dissociated cells. They classify the tissue elements as follows:

(1) *Epithelia*, which they claim are syncytial—both that covering the external surface of the sponge, and that lining the canal system. The former is pierced by 'pores' which are sometimes partly occluded by a pore membrane. The nuclei are irregularly distributed, granular, without a nucleolus. The external syncytium or epidermis is divided up into polygonal areas by 'epidermal lines' which are interpreted as thickenings of the cytoplasm. That they are not cell-boundaries is shown by the fact that they are not correlated with the distribution of nuclei, and that they do not stain selectively with silver nitrate.

This remarkable result is borne out by Penney (1931), who

found that in four fresh-water species (unnamed) investigated by him both epithelia were syncytial. In this case epidermal lines were not seen. There seems no reason to believe that either he or Tuzet are making a mistake, but on the other hand such an important difference between two members of the same order appears *prima facie* unlikely. The question clearly requires further investigation.

(2) Choanocytes, which may always be recognized by their containing a few small, bright red granules.

(3) Nucleolate cells. Sluggish amoeboid cells with a large conspicuous nucleolus and several large orange inclusions. These may be safely homologized with the larger granular cells of Tuzet—(7) (ii) in the list given above.

(4) Grey cells, in which the cytoplasm is filled with numerous small grey granules, staining deeply with methylene blue. In spite of the fact that they possess no nucleolus Tuzet homologizes these with her first type of granular cell.

(5) Globoferous cells, almost exactly similar to those described by Tuzet.

(6) Rhabdiferous cells, which are particularly abundant near the epithelia. They are very elongated and contain numerous rod-like or fibre-like inclusions which stain very readily.

(7) Fibre cells, which are even more narrow and elongated and are only found immediately beneath the epithelia.

They also describe scleroblasts, fibroblasts and sexual cells, whose nature and homology present no difficulty.

They maintain that all the mesenchyme cells are connected together by fine protoplasmic processes, a view that is supported by de Laubenfels (1932). This author in the course of a histological investigation of *Iotrochota birotulata* (a member of the same family as *Microciona*) lays great stress on the importance of the hyaline intercellular ground substance, whose consistency varies from that of water to that of cartilage, and which acts as a scaffolding for the mesenchyme tissues whose cells are widely separated from each other as in *Proterospongia*. The different cell types of *Iotrochota* are easily distinguished by the colour of their inclusions. He describes:

(1) Amoebocytes 'A', with large dark purple granules and no nucleolus. Homologized with nucleolate cells of Wilson and Penney.

(2) Amoebocytes 'B', exactly similar except that the granules are pale green. Perhaps correspond to grey cells.

(3) Amoebocytes 'C', with emerald granules, confined to the superficial layer. Perhaps equivalent to rhabdiferous cells. Also globoferous cells and choanocytes.

Galtsoff (1925) has described the cell elements in dissociated preparations of *Microciona*, but his account has been superseded by the later and fuller one given by Wilson and Penney. According to these authors Galtsoff's pinacocytes are shreds of epithelial syncytium, his choanocytes are wrongly identified, and his archaeocytes, collencytes and desmacytes are respectively their nucleolate, grey, and fibre cells.

To sum up the above results, it appears that the cells of these sponges fall into three categories. Firstly, those whose function and homology is obvious and which are recognizable in all species examined, e.g. scleroblasts, fibroblasts, globoferous cells, choanocytes, germ-cells. Secondly, there are cells that have been only once described and whose equivalents cannot be recognized in other species, such as stellate cells, rhabdiferous cells, &c. Finally, there are the granular mesenchyme cells, which in all the species seem to be of two types, but about whose precise equivalence there is some doubt. It would also appear that in the opinion of the above authors the presence or absence of a nucleolus is a point of no great significance.

Our knowledge of the porocytes has always been far from satisfactory. It had previously been held that, once differentiated, they were incapable of division, but Volkonsky (1930 *b*) has shown that in *Clathrina* they are capable of dividing with a normal mitosis, and Prenant (1925), also working on *Clathrina*, claims that they arise, not as Minchin supposed from exopinacocytes, but from amoebocytes. The question as to whether non-calcareous sponges possess true porocytes is still an open one, the minute size of the prosopyles in most forms rendering observation difficult.

With regard to the question of the identification of the

Metazoan germ-layers in sponges, the only recent pronouncement is by Lameere (1926). He refuses to accept the 'inside-out' theory propounded by Balfour and elaborated by Maas and Delage, which supposes that the parenchymula is comparable to a Coelenterate planula, so that after metamorphosis the ectoderm is internal and consists solely of choanocytes, while all the other tissue elements are endodermal. The main, but not the only reason, according to Lameere, for rejecting this explanation is that the endoderm is without exception throughout the Coelenterata a single layer of cells. In spite of the reversal at metamorphosis he prefers the view of Haeckel, Leuckart, and Schulze that the adult sponge is directly comparable to a Coelenterate, the choanocytes being endoderm and the remaining tissues ectoderm.

#### CYTOPLASMIC INCLUSIONS.

During the period under review several workers have investigated the cells of sponges by means of special cytological techniques with considerable success. The first description of cytoplasmic inclusions in sponges was given by Hirschler (1914), working on *Spongilla fluviatilis*. He observed in the choanocytes a box-like Golgi apparatus in the distal part of the cell (i.e. at the opposite end from the nucleus); the basal granule of the flagellum appeared to be inside it. It consisted of several scales or plates which stained black after prolonged osmication and were not bleached by turpentine. A somewhat similar Golgi apparatus was seen in the amoebocytes: it consisted of several straight or curved rods distributed round the nucleus. According to Hirschler these represent the 'chromidia' described by Jörgensen. By Sjövall's method he was able to detect mitochondria. In both choanocytes and amoebocytes they were scattered throughout the cytoplasm: in the former they were seen in the flagellum but not in the collar. Gatenby (1920a) gives a very similar account of both these structures in the choanocytes of *Grantia compressa*. This author in a later paper on the same species (1927) mentions a peculiar process whereby in certain regions of the sponge the comparatively large mitochondrial granules of the choanocytes undergo fragmenta-

tion, being broken up into about fifty minute granules. The significance of this remains obscure.

Tuzet (1932) in the course of her histological description of *Reniera* deals fully with the cytoplasmic inclusions of the various cells. (The observations of this and other authors on the cytoplasmic inclusions of the germ-cells will be more conveniently dealt with below, in connexion with gametogenesis.) In almost all the cells there is a single annular dictyosome, usually situated close to the nucleus, and consisting of a chromophil cortex surrounding an internal chromophobe mass. The only exceptions to this rule are the granular cells which have two such dictyosomes closely united together, and some of the stellate cells which have two, in this case widely separated. She differs from Hirschler in describing the Golgi apparatus of the choanocytes as being situated beneath and not around the basal granule. Mitochondria were recognized in all the cells: in some cases they are in a single group, in others in two groups or dispersed. In the amoebocytes the mitochondria are at first somewhat scanty, but become plentiful as the cell ages; this phenomenon has also been recorded in *Grantia* by Gatenby.

It has been held by many authors that one of the functions of the mitochondria is intracellular digestion, and two authors claim to show evidence of this in sponges. Volkonsky (1930a) describes digestion in the choanocytes of *Calcarea* as follows. The particle is ingested in the upper part of the cell, just outside the base of the collar, and is received into the substance of the cytoplasm. (The old theory that particles are ingested inside the collar is now generally discredited.) A vacuole is formed round it which is at first neutral or slightly acid and later alkaline. At the latter stage mitochondria condense on it and the whole region becomes rich in lipoids. If the food particle is one that contains much lipid matter—e.g. when a sponge is fed on milk—the mitochondria become very swollen and take some time to recover their normal form. Pourbaix (1933), in studying the digestion of bacteria by the amoebocytes of *Reniera simulans*, notes that the bacterium lies in an alkaline vacuole which is surrounded by a more or less complete ring of mitochondria.

The structures at the apical end of the choanocytes have given rise to some discussion, the real issue at stake being whether or no the Golgi apparatus and the parabasal body are in this case synonymous. The same question has of course been much debated with reference to the flagellate Protozoa. Volkonsky (1929a) describes a juxta-flagellar structure in the choanocytes of *Clathrina*, *Sycon*, and *Leucandra* which he calls the 'corps apical'. Its form is somewhat variable but always approximates to a chromophil cap resting on a chromophobe hemisphere. It is destroyed by alcohol or acetic fixatives, but he maintains that it does not stain with Janus green and therefore cannot be the Golgi apparatus. It divides at or before prophase. He somewhat tentatively suggests that it is the homologue of the parabasal of flagellates. Elsewhere (1929b) he maintains that it was this 'corps apical' and not the Golgi apparatus that Hirschler saw, and that the latter is represented by a 'zone de Golgi' in the middle part of the cell. In a later paper (1930b) he definitely states that the corps apical is equivalent to the parabasal. According to this account which is based on a study of *Clathrina* all the cells have a 'cinétide', consisting of centrosome and parabasal, the choanocytes possessing a flagellum in addition. The parabasal consists of a chromophil plate resting on a chromophobe mass, the former being always attached to the centrosome which, in the case of the choanocytes, may be differentiated into two regions, the mastigosome and the parabasosome. In certain cells such as scleroblasts, amoebocytes and porocytes the parabasal divides precociously so that there are normally two in each cell.

Tuzet (1931), on the other hand, in describing the parabasals of the choanocytes and other cells of *Reniera* and *Hymeniacidon*, holds that the chromophil region does stain with Janus green and that the parabasal is undoubtedly the Golgi apparatus. There is no doubt that both authors are discussing the same structure, since both give identical accounts of its behaviour during oogenesis. The position is, therefore, that the body that Hirschler and Gatenby identified as the Golgi apparatus Tuzet accepts as such, but claims that it is also the parabasal; Volkonsky agrees that it is the parabasal, but denies that it is the

Golgi apparatus. Quite recently Duboscq and Tuzet (1934) have published a detailed study of the Golgi apparatus and parabasal in several calcareous forms. It would seem to add convincing support to the theory which supposes the two structures to be homologous or even identical. These authors claim to have followed the history of the numerous dictyosomes seen in the ovum and early blastomeres, which become differentiated in the amphiblastula into the typical Golgi apparatus of the granular cells and the typical parabasal of the flagellated columnar cells. Furthermore, during the development of a flagellum by the cells of the placental membrane, its Golgi apparatus became gradually transformed into a structure closely resembling the parabasal of the choanocytes. Cell organs of a nature intermediate between parabasal and Golgi apparatus were also seen in scleroblasts and stellate cells.

It is worth noticing that de Saedeleer (1930) records a parabasal in *Codosiga* and other *Craspedomonadina* (Choanoflagellates) precisely similar to that observed in the choanocytes of sponges.

Volkonsky (1930b) describes an interesting phenomenon with reference to the parabasal of the choanocytes of *Clathrina*. It appears to undergo a regular cycle in which it leaves the centrosome and travels up to the proximal end of the cell near the nucleus, where it swells up and disappears. A new parabasal is regenerated from the centrosome: this also occurs when, as sometimes happens, the parabasal fails to divide during cell division. A very similar cycle has been observed by Lwoff and Lwoff (1930) in the blepharoplast of *Leptomonas ctenocephali*. Duboscq and Tuzet (1934), however, working on *Clathrina* and other *Calcarea*, find no evidence of the existence of this periodical change.

The granules that are found in the cells of *Clathrina coriacea*, particularly in the porocytes and to a lesser extent in the amoebocytes, are described by Teissier and Volkonsky (1930). They regard them as cell inclusions *sui generis*, being neither vacuome nor chondriome. These granules may be either white or yellow, and the sponge as a whole is coloured accordingly. They are soluble in alcohol except after fixation,



which renders them insoluble and turns them a yellowish-brown colour. These authors consider them to be composed of an oxylipoid material with a small amount of protein. Several other authors have mentioned pigment granules in different species, but in insufficient detail to be able to compare them with those of *Clathrina* described by Teissier and Volkonsky.

#### GAMETOGENESIS.

All forms in which gametogenesis has been thoroughly investigated, viz. *Reniera*, *Sycon*, *Grantia*, and *Spongilla*, appear to be hermaphrodite. In all cases, however, stages in spermatogenesis are much rarer than stages in oogenesis: in twenty-five individuals of *Grantia* sectioned by Gatenby (1920a) only one showed spermatogenesis. And it appears that it is only in littoral species that female elements are at all common. According to Burton (1928) deep-sea forms reproduce almost entirely asexually. Among Hexactinellids there are only two cases of ova recorded, four of embryos, and none of spermatozoa: reproduction seems to be effected mainly by buds. Among deep-water Tetraxonida ova, spermatozoa, and early embryos are quite unknown. Apparent highly developed 'embryos' are common, but there is strong reason to suppose that they are produced asexually from aggregations of amoebocytes.

The origin of the germ-cells has given rise to considerable controversy. Haeckel held that they arose from choanocytes, but Schulze, Poléjaeff, and Maas put forward evidence that they were derived from amoebocytes, and under the influence of Weismannian doctrines this view triumphed to such an extent that Minchin, writing in 1900, does not even mention the other theory. Dendy (1914), however, disinterred Haeckel's 'heresy' and stated his belief that in *Grantia* the germ-cells arose from choanocytes. Gatenby (1920a) supported this view and brought forward further convincing evidence. Tuzet (1930a and 1930b) considered that in *Cliona* and *Reniera* the germ mother-cell was an amoebocyte, but in a later paper on *Reniera* (1932) she has changed her mind and holds that Haeckel's theory is true for siliceous as well as calcareous sponges. There seems to be little doubt that the germ-cells are derived from choanocytes

which sink down into the mesenchyme, though they pass through a stage in which they bear a close enough resemblance to amoebocytes to have deceived the earlier workers.

As already stated, stages in spermatogenesis are rare, and the process still remains rather a mystery. Quite recently Gatenby (1927) was able to write that 'except for Görich's paper which is cytologically imperfect, the questions surrounding the appearance of sponge spermatozoa are largely unanswered'. The 'sperm-morulae' described by Dendy (1914) in *Grantia* both Gatenby (1920a) and Bidder (1920) believe to be a parasitic or endozoic alga. In this paper Gatenby describes nests of spermatids lying between the flagellated chambers and surrounded by an envelope of pale cubical cells. He also mentions having seen groups of cells which he thought were probably spermatocytes. In a supplementary account (1927) he considers that in *Grantia* spermatogenesis may take place in either of two ways—that previously described in which pockets of spermatocytes are formed in the mesenchyme, or else by the rapid conversion of all the cells of a flagellated chamber into spermatocytes whereby the collar and flagellum are lost, the nucleus becomes reticulate, and the mitochondria fragment into smaller granules.

Tuzet (1930a) describes spermatogenesis in *Reniera*. The primordial germ-cell undergoes amitotic division whereby one of the daughter nuclei receives the nucleolus, the other most of the chromatin network. Of the two cells thus formed, that possessing the nucleolus undergoes no further development except that its cytoplasm stretches so as to embrace the other cell: it is alluded to as the cover cell. The second cell divides repeatedly, forming sixteen spermatogonia which undergo a normal maturation including a reduction division (from sixteen to eight chromosomes). In the spermatid the mitochondria first aggregate into four spherules: these subsequently adhere together to form the middle piece of the spermatozoon. Elsewhere (1931 and 1932) she follows the behaviour of the dictyosome (parabasal) in the spermatogenesis of *Reniera* and *Hymeniacidon*. It divides with each cell division so that there is one in each spermatid: precocious division in the primary spermatocytes,

however, usually means that at this stage there are two dictyosomes in each cell.

Del Rio Hortege and Ferrer (1917) published a very curious account of the spermatogenesis of *Reniera permollis* and *Reniera rosea*. Weill (1926) has shown that it was based on a misconception, and that the so-called spermatozoa were really the nematocysts of a parasitic Scyphozoan, *Nausithoe* (= *Stephanoscyphus mirabilis* Allman and *Spongicola fistularis* Schulze), that has been recorded from several species of sponges. Half-digested nematocysts were described as stages in spermatogenesis, while the 'immature spermatozoa' seem to have been genuine spermatozoa of this or some other sponge.

All authors are now agreed that sponge spermatozoa are of an ordinary filiform type, like those of most other animals.

The accounts of oogenesis—mainly by the same authors—are more complete. Dendy's (1914) description of the process in *Grantia* has been confirmed and amplified by Gatenby (1920a) and Duboscq and Tuzet (1933b) except for the interpretation of certain granules in the cytoplasm that have given rise to some discussion and will be dealt with later. The following account is derived from these three papers. A choanocyte loses its collar and sinks down into the mesenchyme, where it proceeds to grow into an oogonium, the flagellum being retained for a while and then lost. There are at least two generations of oogonia, the first large (12–15 $\mu$  in diameter), the second small (under 8 $\mu$ ). Jörgensen had described these two generations but had transposed their order. When the first generation reaches the spireme stage prior to division it migrates back towards the flagellated chamber and undergoes division among the choanocytes. The oogonia of the second generation then migrate back into the mesenchyme to become oocytes. There appears to be an ordinary reduction division, but there is some disagreement as to the chromosome number in *Grantia*. Duboscq and Tuzet regard twenty-six for the diploid number as most likely. In the oocyte, which grows to a considerable size, Golgi bodies, mitochondria, and very delicate yolk-granules can be recognized. It is clearly differentiated into a hyaline non-vacuolated ecto-

plasm and a frothy and granular endoplasm: this can be observed even in quite young oocytes. It possesses no apparent polarity nor anything that can be interpreted as organ-forming substances. During its growth not only is yolk pushed into it by the surrounding cells, but it appears to ingest whole amoebocytes containing yolk, a process that is apparently unique in the animal kingdom. Most authors imply, and Dendy (1914) and Duboscq and Tuzet (1932) explicitly state, that all oogonia or oocytes in one individual are always at practically the same stage in development.

In *Reniera*, according to Tuzet (1932), the process is simpler. The choanocyte, having been transformed into a germ mother cell, grows directly into an oocyte without any intervening oogonial generations.

Several authors have described chromophil and osmiophil bodies in the cytoplasm of the oocyte, but they disagree widely as to their nature and origin. Both Dendy and Jörgensen had described the extrusion of chromidia into the cytoplasm during oogenesis. Gatenby, in his study of *Grantia* (1920*a* and 1920*b*), accepted this explanation first with hesitation, but later whole-heartedly. In these papers he expressed the view that the extruded granules were probably mitochondria, although their fixing reactions were not quite the same as those of normal metazoan mitochondria. In his later paper (1927) he felt more confident as to their mitochondrial nature, since he had been able to follow their history and detect them in the flagellated cells of the amphiblastula. But the situation was complicated by the fact that he found very similar granules in the oocytes of *Sycon* without any signs of nucleolar extrusion. These bodies can be traced back to a single juxtanuclear structure in the young oocyte which undergoes repeated division. Now three later descriptions have been published of a body which is single in the young oocyte and multiplies during its growth—in *Clathrina* by Volkonsky (1930*b*), who states that it is the parabasal, and in *Reniera* and *Hymeniacidon* by Tuzet (1931 and 1932), who calls it the dictyosome which, it will be remembered, she (but not Volkonsky) regards as being identical with the parabasal. Finally, Duboscq and Tuzet (1933*b*) describe

nucleolar emission in *Sycon* but declare that the granules thus formed are not mitochondrial.

It seems impossible to form an impartial judgment as to the relative merits of these views, especially as the later authors when describing phenomena that are inconsistent with earlier results do not offer any explanations of the inconsistency. It is also very uncertain to what extent all these bodies belong to the same type. One can only hope that future work on these and other genera may elucidate the problem.

#### FERTILIZATION AND EMBRYOLOGY.

The first adequate description of fertilization in a sponge was given for *Grantia* by Gatenby (1920a, 1920b, 1927). As he points out, however, previous workers had seen stages in fertilization without realizing their significance, Jörgensen having mistaken it for the expulsion of nuclear material and Dendy for phagocytosis. Gatenby's account of the process is as follows.

The spermatozoon is carried into a flagellated chamber beneath whose lining lies an oocyte. It does not find its way directly to the oocyte, however, but only by means of an intermediary carrier cell. Accordingly it enters one of the choanocytes lining the flagellated chamber: as a rule the choanocyte selected is one that lies directly over the oocyte that is to be fertilized. In fact, the spermatozoon usually gets so close to its ultimate objective that it is held that this must be a case of chemotaxis. Occasionally it makes a 'bad shot' and enters a choanocyte some distance from the oocyte; this seems to happen more often in *Sycon* than in *Grantia*. After the entry of the sperm the choanocyte undergoes considerable changes, whereby it is converted into a carrier cell; the flagellum and collar are lost, the nucleus sometimes takes on a reticulate structure so as to resemble that of an amoebocyte, the Golgi apparatus and mitochondria are retained but become much more difficult to stain, and finally the cell rounds itself off and sinks down into the mesenchyme from its place in the general epithelium. In most cases this suffices to bring it into contact with the oocyte, otherwise it travels through the mesenchyme till it reaches it. A few cases were observed in which fertilization took

place on that side of the oocyte remote from the gastral epithelium: presumably the carrier cell had wandered right round. Meanwhile changes have taken place in the spermatozoon. It first loses its tail, and then the nucleus and middle piece swell considerably till it becomes converted into what is called by French authors the 'spermiokyste'. This is a 'cottage-loaf' structure consisting of the nucleus and middle piece, both more or less globular, and tipped by the crescentic acrosome which has, however, become much more chromophobe. In this state it lies in a vacuole of the carrier cell. When the latter comes up against the oocyte protoplasmic continuity is established at the point of contact and the sperm flows passively into the oocyte, its path of entry being visible for some time as a chromophobe, streak-like vacuole. Its entry provides the stimulus for maturation of the ovum and two polar bodies are given off. Meanwhile the middle piece of the sperm breaks up, the male pronucleus approaches that of the ovum and both swell to the same large size prior to fusion. As regards the fate of the carrier cell Gatenby at first thought that it wandered off through the mesenchyme and eventually degenerated, but later (1927) he affirmed that it returned to its original situation and took on once more the form of a choanocyte.

Duboscq and Tuzet's (1932 and 1933b) account of fertilization in *Grantia* and *Sycon* is mainly confirmation of the above results. They did not, however, see in *Sycon* the spermatozoa with the long middle piece described by Gatenby, and suggest that they must be degenerate or belong to some other organism. They describe the spermatozoa as having a conical head and a disc-shaped middle piece. In both genera the spermatozoa appeared to enter the choanocyte within the base of the collar, whereas food particles are ingested outside its base: this implies an actively swimming sperm. They disagree with Gatenby's finding that the carrier cell returns to the flagellated chamber to become a choanocyte, and hold instead that it hypertrophies and then degenerates in the mesenchyme. During the early stages of cleavage of the ovum these hypertrophied cells are frequently to be seen in its vicinity.

Tuzet (1930b and 1932) has investigated the process in the

siliceous sponges *Cliona* and *Reniera*. She finds it very similar to that described in the calcareous forms with one important difference—the carrier cell is one of the amoebocytes in the neighbourhood of the ovum and not a choanocyte. This distinction between the two classes is correlated with structure and is indirectly confirmed by some results obtained by Pourbaix (1934) who investigated the feeding methods of various species. She confirmed and elaborated the distinction first suggested by Metschnikoff, that in *Calcarea* ingestion of food particles is accomplished by the choanocytes, in *Silicea*, particularly those with a highly evolved type of canal system, by the amoebocytes. Since spermatozoa are up to a point comparable to food particles it is very probable that the difference between Tuzet's and Gatenby's results is due to the anatomical differences between the two classes of sponge.

The essential features of sponge embryology, particularly among the *Calcarea*, have been known for some time, so that recent work has been only concerned with points of detail. Gatenby (1920a) describes the cytology of the amphiblastula of *Grantia*. There is no segregation of mitochondria or yolk into certain areas during cleavage, the difference between the two main types of cell in the amphiblastula being due to a difference in the nature of the ground-cytoplasm itself. As has already been mentioned, there is no apparent polarity in the ovum. In most cases the flagellated hemisphere of the larva faces the nearest flagellated chamber, and this being the aspect on which fertilization usually takes place it is possible that the latter process may be the sole determinant of the position of the embryonic axis, but there are just enough exceptions to render this causal connexion somewhat doubtful. The cells of the amphiblastula are described as follows:

(1) Flagellated columnar cells which possess a dense mass of yolk at their inner end and mitochondria scattered throughout the cytoplasm. The nucleus is small and deeply staining and includes a karyosome.

(2) Granular cells, with rather less yolk and about the same amount of mitochondria as the flagellated cells. Their nucleus is larger and paler, but also contains a karyosome.

(3) Mesenchyme cells, of which there are only two or three. They contain very little yolk but are crammed with mitochondria. They are derived from flagellated cells that sink in to the centre of the larva.

(4) Duboscq and Tuzet (1933a) describe in the larva of *Sycon* four peculiar cells situated in the third row of flagellated cells counting from the equator. They are symmetrically arranged so as to form a Greek cross when the larva is viewed in transverse section. They are considerably larger than the flagellated cells, the nucleus is central with a chromatic ring immediately distal to it, and there are large granules at the base. It is conjectured that they may be light-perceptive cells. Their ultimate fate is unknown.

Gatenby and King (1929) state that the embryo of *Grantia* is surrounded by a definite placental membrane composed of flattened cells containing osmiophil granules. They are apparently derived from amoebocytes. Those cells adjacent to the granular hemisphere of the larva are more granular than those next the flagellated hemisphere. Gatenby (1920a) had previously noticed that the surrounding maternal cells often penetrate in among the granular cells of the larva so that the line of demarcation between the two tissues may be ill defined. Duboscq and Tuzet (1933a) interpret this as a means of feeding the larva: whole cells filled with yolk penetrate into it, much as in the growth of the oocyte. According to these authors the discharge of the larva is heralded by the acquisition of flagella by the cells of the placental membrane. The cavity containing the larva then becomes confluent with the nearest flagellated chamber, and the larva passes out. Usually a great number of larvae are released simultaneously, and according to Orton (1929) this is followed by the break-up of the sponge, so that *Grantia* normally dies from over-reproduction. O. Jorgensen (1918) has followed the history of the larvae. They swim freely for twenty-four hours, then settle to the bottom and overgrowth of the granular cells begins. This lasts about two days, by which time fixation is well in progress. This author states that all larvae are ripe at the same time—early September—but Dendy (1914) quotes Orton as saying that there are two breeding



seasons, June and October, while according to his own observations embryos are released throughout the summer. It seems rather doubtful that *Grantia* has a fixed breeding season.

Only two recent papers have been published dealing with the embryology of non-calcareous sponges. Vaney and Allemand-Martin (1918) describe the larva of *Hippospongia* which is released at Tunis in the early summer. It is ovoid, the broader (posterior) end being marked by a dark ring. The surface is ciliated all over, but the cilia in the area circumscribed by this ring are extremely long. The cells of the ring are loaded with pigment granules that are periodically discharged. Under the epithelium lies a syncytial layer and beneath this a solid core of large fusiform cells. Wilson (1932) has investigated the 'inversion of the germ-layers' in the Monaxonid *Mycale* and finds that, although there is an outward migration of the amoebocytes so that the larval epithelium (now internal) develops into the choanocytes, there is no evidence for the process described by Delage whereby, in order to effect this inversion, the epithelial cells were supposed to be engulfed and subsequently liberated by the amoebocytes.

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# The Early Embryonic Development of *Rhodnius prolixus* (Hemiptera, Heteroptera).

By

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With Plate 5 and 11 Text-figures.

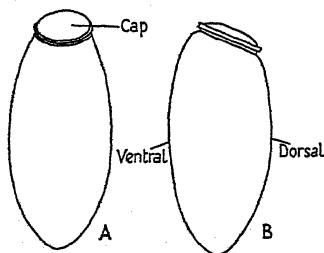
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## INTRODUCTION.

*Rhodnius prolixus* Stal is a blood-sucking bug of the family Reduviidae, and is a native of South America. A great deal of work has been done on the nymphal and adult stages (Buxton, 1930; Wigglesworth, 1931, 1933, 1934), and it has proved an admirable experimental animal. For this reason it seemed desirable to work out in detail its development within the egg.

A culture of *Rhodnius* was obtained from Professor P. A. Buxton, of the London School of Hygiene and Tropical Medicine, and was bred by the methods described in his paper (Buxton, 1930). The insects were fed at intervals on rabbit's blood. The adults were kept at a constant temperature, 21° C., and the blotting-paper among which they lived was examined frequently for eggs. For the earlier stages the age of the eggs was known to within 1 hour. For later stages their ages were not quite as accurately known in all cases. The eggs were incubated under constant conditions of temperature and humidity for varying lengths of time from 1 hour up to 5 days. This was considered very important, for it is well known that temperature and humidity greatly affect the rate of development. Previous workers have not incubated their eggs under controlled conditions, with the result that they have been unable to produce accurate time-tables of development. Even under conditions of constant temperature and humidity there was slight individual variation in the time taken to develop to any given stage (see Table 1, p. 5).

The eggs were kept at 21° C. and 90 per cent. relative humidity; the duration of the embryonic period of controls under these conditions was approximately 29 days. There was a slight variation in the time taken to hatch, but it was never longer than 30 days. Eighty-five per cent. of the eggs hatched successfully. The remainder all developed, but the nymphs died in the process of emerging. This is known to happen with



TEXT-FIG. 1.

Eggs with chorion intact. A. Dorsal view. B. Lateral view.

other bugs, for instance with *Cimex lectularius*. The temperature chosen for incubation was one at which development proceeded very slowly. The humidity was high since *Rhodnius* eggs are known to dry up readily.

Of all the eggs examined by means of sections (over 130), only one was found which had failed to develop.

The eggs of *Rhodnius* vary somewhat in size and shape. They are elongate oval, with a cap at the anterior end (Text-fig. 1). They are 1.5 mm. by 0.8 mm. approximately. The dorsal surface is slightly flattened, while the ventral side is convex. The cap slopes towards the dorsal side. As the eggs were fixed with their chorions intact (except in some cases for removal of the cap), the yolk solidified in the shape of the egg. This was of great assistance in section cutting, as it was possible to tell from the sections which side was dorsal and which ventral, and whether the orientation was correct or not. This fact was of importance when following the stages in blastokinesis.

The chorion of the egg is sculptured; in life it is hard and fairly

brittle. The eggs are pale pink; the pigment is contained in the yolk and it shows through the transparent chorion.

#### METHODS.

The eggs were fixed in Carnoy, Bouin, or hot alcoholic Bouin. The first fixative gave the best results. When using Bouin the caps of the eggs were removed with a needle to ensure penetration of the fixative. After the eggs had been kept in 90 per cent. alcohol for about 3 days the chorion was dissected off with needles. The alcohol made the chorion sufficiently brittle for it to be easily removed without damaging the underlying yolk and embryo. Slight shrinkage of the yolk facilitated this process. Successive stages in the development of the embryonic rudiment were drawn as a whole while the eggs were in 90 per cent. alcohol; staining of the whole mounts was found unnecessary because the pale embryo showed up against the pigmented yolk. Eggs for sectioning were double embedded in celloidin and wax by Newth's method.

Sections were cut  $8\mu$  thick and were stained with a variety of stains. Ehrlich's haematoxylin and eosin were mainly used. Alcoholic haematoxylin, Giemsa, Leishman, safranin, Küll's, and Volkonsky's staining methods were also tried.

The earliest stages before cleavage took place were extremely difficult to stain. In the vast majority of sections no nuclei were visible. This may be due to the breaking up of the nuclei on fixation. Various bodies, possibly consisting of chromatin, appeared scattered among the cytoplasm surrounding the yolk-spheres.

In all the later stages the cytology appeared to be reasonably good. The method of double embedding usually ensured the easy sectioning of the yolk.

#### The Structure of the Egg shortly after Oviposition.

The structure of the egg half an hour after being laid was that of a typical insect egg-cell. A single nucleus with its surrounding cytoplasm was situated at the centre of a large quantity of yolk. Strands of cytoplasm radiated out between the yolk-globules and connected the central cytoplasm with a peripheral

layer, which lay immediately underneath a very thin vitelline membrane. The yolk during life was liquid and appeared to be composed of a large number of spheres. After fixation the spheres were still apparent in the earliest stages, and were separated from one another by a very thin layer of cytoplasm. At all stages the yolk stained very readily with any of the methods used. After 2 hours' incubation a nucleus and cytoplasm were found to have moved from their central position and to be nearer the surface. Possibly this was the egg nucleus about to undergo maturation. I was not able to find any complete nuclei in eggs of between 2 and 11 hours old. A cytoplasmic network was visible, and some bodies which stained like chromatin were seen scattered through the cytoplasm. A combination of several fixatives and staining methods gave very similar results. Possibly the nuclei at this stage are very fragile and easily broken up by fixatives. In eggs more than 12 hours old the cleavage nuclei were visible, and they steadily increased in number as time went on.

#### Cleavage and Blastoderm Formation.

After 12-13 hours of incubation the first cleavage occurred. At 18 hours there were between 4 and 8 cleavage nuclei (Text-fig. 2). By 25 hours there were at least 32 nuclei. These early cleavage nuclei lay towards the anterior end of the egg, and they had not moved far from the centre of the yolk. When the number of nuclei reached 32, they had begun to migrate to the periphery, fig. 1., Pl. 5. As in most other insects, the whole egg is a syncytium at this stage and remains such until the formation of the blastoderm is completed. The cleavage nuclei were rounded and lay in an irregular mass of cytoplasm which was continuous with a thin layer of cytoplasm round the yolk-spheres (fig. 1., Pl. 5). Their position in the egg was similar to those of *Pieris* at the same stage (Eastham, 1927), but they never showed the conspicuous comet-like appearance as shown by the nuclei of that species. The chromatin in the nuclei of *Rhodnius* does not stain deeply until blastoderm formation has begun.

There was no cleavage of the yolk in *Rhodnius*. The

structure of the yolk changed as development proceeded. At first it was a mass of small spheres of  $40\mu$  in diameter. Later, when the cleavage nuclei had reached the periphery and there was no longer a layer of cytoplasm round the yolk-globules, these seemed to run together, but their continuity was broken by large spaces. These spaces were presumably due to some ether soluble substance having been dissolved out during the technique of celloidin embedding.

Not all the cleavage nuclei migrated to the periphery. Some remained behind and gave rise to the yolk-nuclei (fig. 4, Pl. 5). Those which migrated moved through the yolk with their surrounding cytoplasm and came to lie in the peripheral layer of cytoplasm underneath the vitelline membrane. At first they were situated at a considerable distance from one another though they were connected by the peripheral cytoplasmic layer (fig. 2, Pl. 5). Soon the nuclei divided tangentially, so reducing the space between each nucleus (figs. 2 and 3, Plate 5). As a result of the first few cleavage nuclei having been formed in the centre of the anterior half of the yolk, it was in the anterior part of the egg that the nuclei first reached the periphery. It was several hours later before they reached the posterior surface. In this *Rhodnius* resembled *Pieris* and the beetle *Europe terminalis* (Paterson, 1931), but differed from the holly tortrix moth (Huie, 1917), and the beetle *Hydrophilus* (Heider, 1885).

After 30 hours' incubation a large number of the cleavage nuclei had migrated to the periphery of the egg and had taken up their position near the surface. The formation of the blastoderm was very similar to that described for *Pieris* (Eastham, 1927) and for the beetle *Europe* (Paterson, 1931). The peripheral cleavage nuclei increased in number by tangential divisions. The cytoplasm belonging to adjacent nuclei became continuous, forming a syncytial layer (fig. 3, Pl. 5). The external surface of the egg remained smooth, except at the posterior end where the nuclei protruded beyond the surface of the egg as they do in some insects (fig. 6, Pl. 5) (Eastham, 1927; Marshall and Dernhehl, 1905; Nelson, 1915). The vitelline membrane was not distinct at this stage. The syncytial layer



was very thin and gave the appearance of being stretched over the egg surface. Cell-walls dividing each nucleus and its cytoplasm from its neighbours began to appear after 50 hours. They were only distinct in the parts of the blastoderm destined to form the embryonic rudiment. The remaining, extra-embryonic, blastoderm became clearly separated from the yolk, but the limits of the cells were difficult to distinguish. The nuclei in this part became flattened and elongated very early, giving the characteristic appearance of serosa cells.

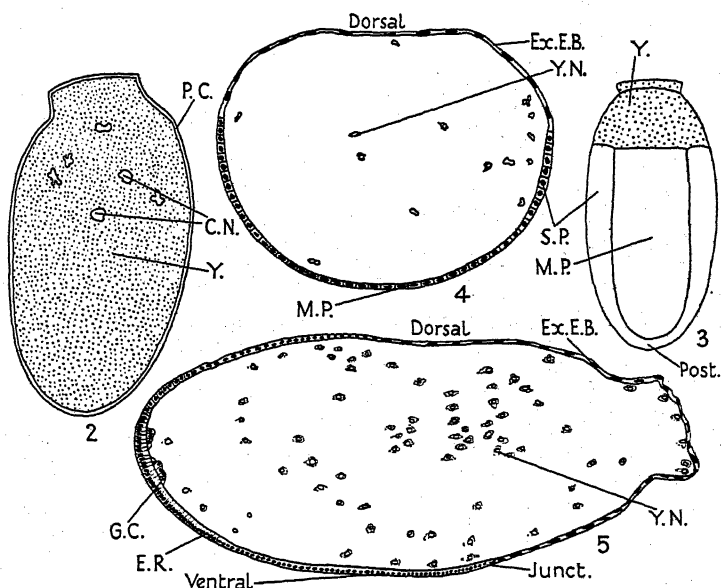
The blastoderm was complete anteriorly before it was formed at the posterior end of the egg. Differentiation of the blastoderm began on the ventral side of the egg. The ventral and lateral portions of the blastoderm became differentiated into cubical cells, while the dorsal and lateral parts remained covered by flattened epithelium (Text-fig. 4). The cubical cells gave rise to the embryonic rudiment, while the flattened cells became the extra-embryonic blastoderm.

#### Vitellophages.

The yolk-nuclei were derived from cleavage nuclei which remained behind instead of migrating to the edge of the yolk. I have not found another example of an exopterygote insect in which the origin of the vitellophages was definitely stated to be from cleavage nuclei (fig. 6, Pl. 5). Some nuclei migrate nearly to the periphery before lagging behind the others. The vitellophages were large and conspicuous at first, and they stained more deeply with haematoxylin than did cleavage nuclei of the same age. They multiplied by mitosis up to the age of 2 days. After that age no evidence of mitosis was seen, though it was not uncommon to find yolk-nuclei in pairs or in threes, giving the impression that they might have recently divided. After formation of the embryonic rudiment, large numbers of cells were given off into the yolk. There they gradually disintegrated.

#### Formation of the Germ-band.

The differentiation of the blastoderm into embryonic rudiment occurred on the ventral side of the egg. The blastoderm



TEXT-FIG. 2. Diagrammatic longitudinal section of egg after 18 hours of incubation. *C.N.*, cleavage nuclei; *P.C.*, peripheral layer of cytoplasm; *Y.*, yolk.

TEXT-FIG. 3. First appearance of embryonic rudiment. Ventral view, drawn from whole mount after 76 hours of incubation. Diagrammatic.  $\times 45$ . *M.P.*, middle plate; *Post.*, posterior end of egg; *S.P.*, side plate; *Y.*, yolk.

TEXT-FIG. 4. Transverse section of egg at the time of first appearance of embryonic rudiment. Mag. 70. Semi-diagrammatic. *Ex.E.B.*, extra-embryonic blastoderm; *M.P.*, mid-ventral portion of embryonic rudiment; *S.P.*, ventro-lateral portions of embryonic rudiment; *Y.N.*, yolk nucleus.

TEXT-FIG. 5. Longitudinal section of egg showing early embryonic rudiment and the germ-cells.  $\times 65$ . Diagrammatic. *E.R.*, embryonic rudiment; *Ex.E.B.*, extra-embryonic blastoderm; *G.C.*, germ-cells; *Junct.*, junction of embryonic with extra-embryonic blastoderm.

covering the egg ventrally, laterally, and posteriorly, was transformed into cubical epithelium; the remainder of the blastoderm was made of very flattened epithelium. The cells of the cubical epithelium or ventral plate became thickened along two ventro-lateral areas (Text-figs. 3 and 4). These areas were separated at the anterior end of the yolk by a median portion of ventral plate which was made of smaller, less cubical, cells with more elongated nuclei. These cells were not as flattened as those forming the extra-embryonic blastoderm. The ventro-lateral areas of cubical epithelium ran parallel until they reached the posterior end of the egg. Here they turned towards one another and met. The total effect was that of a U with the open end facing anteriorly, the cells inside the U being smaller. The cubical epithelium then extended its area round the posterior end on to the dorsal surface (Text-fig. 5). This was very similar to the first appearance of the embryonic rudiment in the bug, *Pyrrhocoris apterus* (Seidel, 1924). This stage could be seen in whole mounts because the parallel areas of cubical epithelium, being made of large cells, showed up white against the pinkish colour of the underlying yolk.

Longitudinal sections of this stage (Text-fig. 5) showed a number of small cells with very conspicuous nuclei situated at the posterior pole of the egg. These cells were budded off from the blastoderm and were continuous with it (fig. 8, Pl. 5). I assume that these are the germ-cells of *Rhodnius*, because germ-cells, cytologically similar, have been found in the same position in other insects (Imms, 1925; Hirschler, 1909). Poluszynski (1911) found the same arrangement in a Coccid bug, but apparently the germ-cells in *Pyrrhocoris apterus* (Seidel, 1924) were not differentiated at this early stage. It is interesting to observe that *Pyrrhocoris* does not resemble *Rhodnius* although the two species belong to the same sub-order of the Hemiptera.

In *Rhodnius* the germ-cells made their first appearance between 66 and 76 hours after the eggs were laid. They increased in number by further immigration and by occasional mitosis until they formed a mass of cells projecting inwards into the yolk.

The area of the egg covered by the embryonic blastoderm now began to decrease. The thickened ventro-lateral portions became withdrawn towards the mid-line. The result of this was that the embryonic cells took on a more columnar form, and the cells in the mid-ventral part became columnar too, though they had previously been flattened.

#### Change in Position of the Embryonic Rudiment.

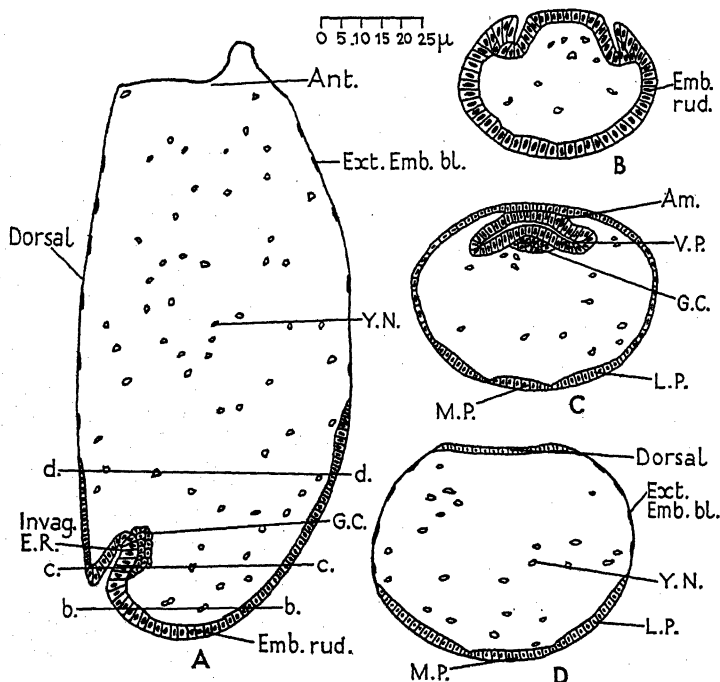
A few hours later invagination of the germ-band began. At a point near the dorsal side of the egg, where the superficial embryonic rudiment curved round the posterior pole, there began a proliferation of cells inwards into the yolk. An intucking of the embryo followed at this point, and proceeded very rapidly (Text-figs. 6 and 7). The intucking took place just dorsally to the mass of germ-cells.

The invagination caused the ventral part of the embryonic rudiment to move towards the posterior end of the egg, since the posterior end of the rudiment was becoming tucked into the yolk. The cells of the extra-embryonic blastoderm became more flattened as this process took place, as they had to cover an increased area of yolk, and their number did not increase. The process of invagination continued until all but the extreme head end of the embryo was surrounded by yolk. The head-end (which was by this time at the posterior pole of the egg) remained in its superficial position for a short time; then it too became surrounded by yolk (Text-fig. 8 A), but it remained flexed towards the ventral side of the egg. The part of the embryonic rudiment other than the head lay very much nearer the dorsal surface of the egg than the ventral.

When the embryo had taken up this new position it had moved through  $180^{\circ}$  and was completely reversed in its relation to the chorion. It lay in a dorsal position with its head facing the posterior end of the egg and with its posterior extremity facing the anterior end. The original ventral surface of the embryonic rudiment was now dorsally placed. This marked the end of the first stage in blastokinesis, which is a characteristic of exopterygote insect development when there is much yolk.

## Change in Form of the Embryonic Rudiment.

The invaginated portion elongated very quickly towards the anterior end of the egg, at the same time becoming very narrow

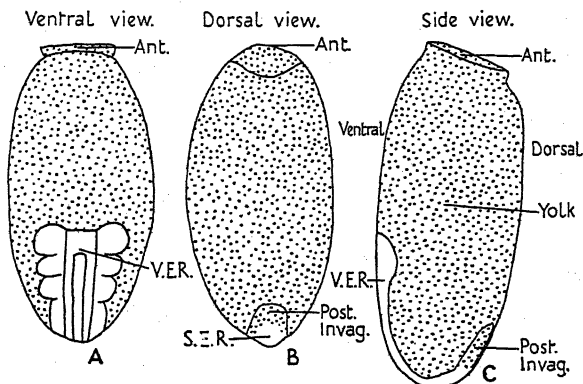


TEXT-FIG. 6.

Longitudinal section and transverse section of embryonic rudiment during invagination, after 80 hours' incubation.  $\times 66$ . Diagrammatic. A. Sagittal section. B. Transverse section at level *bb* on sagittal section. C. Transverse section at level *cc* on sagittal section. D. Transverse section at level *dd* on sagittal section. *Am.*, amnion; *Ant.*, anterior end of egg; *E.R.*, embryonic rudiment; *Ext. E.B.*, extra-embryonic blastoderm; *G.C.*, germ-cells; *Invag. E.R.*, invaginated part of embryonic rudiment; *L.P.*, lateral plate; *M.P.*, middle plate; *V.P.*, ventral plate; *Y.N.*, yolk nuclei.

and nearly cylindrical in cross-section (Text-fig. 8). The germ-cells were carried forward through the yolk, always occupying a position near the extreme posterior end of the embryo

(Text-fig. 8). Invagination and narrowing of the embryonic rudiment took place synchronously, transforming the broad superficial rudiment into a long thin germ-band, almost all of which was sunk into the yolk. The anterior end of the germ-band remained superficially placed longer than the rest of the rudiment. At first it covered quite a large area (Text-fig. 9 A),



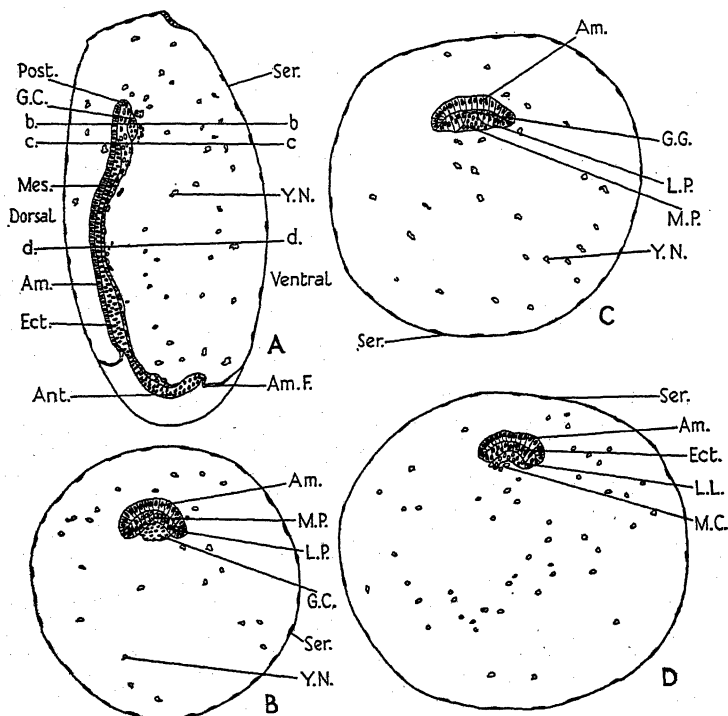
TEXT-FIG. 7.

Drawings of whole mounts showing invagination process.  $\times 46$ .  
 A. Ventral view. B. Dorsal view. C. Side view. *Ant.*, anterior end of egg; *Post. invag.*, posterior part of embryo invaginating; *S.E.R.*, superficial part of embryonic rudiment; *V.E.R.*, ventral part of embryonic rudiment.

but soon its edges contracted (Text-fig. 9 B), causing this part of the rudiment to thicken, and by 90 hours it also had become sunk into the yolk (Text-fig. 8). At this stage, therefore, there was a marked cephalic flexure. This flexure remained until 12 days of incubation. There was no caudal flexure in *Rhodnius* like that which occurs in *Pyrhocoris*, or the bed-bug *Cimex lectularius* (Heymons, 1899).

The method of invagination is shown in Text-fig. 6, and Text-figs. 6 B, C, and D show transverse sections taken at three different levels during the beginning of blastokinesis. There was active division of the cells of the embryonic rudiment during this process. Text-fig 6 C shows the position of the germ-cells in transverse section. They lay below the layer of cells

from which the rest of the embryo was to be developed, and they were next to the main part of the yolk. The invaginated

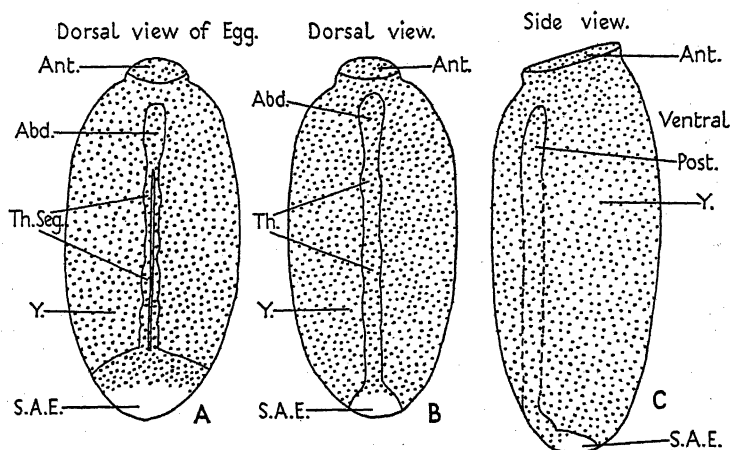


TEXT-FIG. 8.

Longitudinal section and transverse section of embryo immediately after invagination. Diagrammatic. A. Sagittal section after 90 hours' incubation.  $\times 50$ . B. Transverse section same age as (A) along level *bb*.  $\times 90$ . C. Transverse section same age as (A) along level *cc*.  $\times 90$ . D. Transverse section same age as (A) along level *dd*.  $\times 90$ . *Am.*, Amnion; *Am.F.*, amnion fold; *ant.*, anterior end of embryo; *Ect.*, ectoderm; *G.C.*, germ-cells; *G.G.*, gastral groove; *L.L.*, lower layer (mesoderm); *L.P.*, lateral plate (ectoderm); *M.C.*, lower layer cells degenerating in yolk; *Mes.*, mesoderm; *M.P.*, middle plate (mesoderm); *Post.*, posterior end of embryo; *Ser.*, serosa; *Y.N.*, yolk nuclei.

part of the embryo was at first a simple sac made out of cubical epithelium (Text-fig. 6 c). The sac was flattened in a dorso-

ventral plane, and its internal cavity was the future amniotic cavity. The dorsal portion of cubical epithelium was the amnion, while that which was nearest the main part of the yolk was the ventral plate or embryonic rudiment. This invaginated ventral plate was made of much more columnar cells than the



TEXT-FIG. 9.

Diagrams of whole mounts, after 90–100 hours of incubation. Invagination complete.  $\times 45$ . A. Dorsal view about 90 hours. B. Dorsal view slightly older than (A). C. Lateral view of (B). *Abd.*, abdominal part of embryo; *Ant.*, anterior end of egg; *Post.*, posterior end of embryo; *S.A.E.*, superficial anterior end of embryo; *Th.seg.*, thoracic segments; *Y.*, yolk.

original cubical epithelium of the embryonic blastoderm. The cells were now much more tightly packed together, and this caused the germ-band to be thicker but much narrower than before. At first the cells forming the amnion were the same shape and size as those of the embryonic rudiment (Text-fig. 6 c); some time after invagination was completed the amnion cells became elongated and thin anteriorly, while those of the ventral plate grew larger (fig. 9, Pl. 5). The meeting of the amnion folds and the broadening of the embryo in the future head and thoracic regions caused the amnion to be stretched and pulled out until it was a layer of much flattened cells.



### Embryonic Membranes.

The serosa was derived from the cells of the extra-embryonic blastoderm (Text-figs. 5 and 8). After the germ-band had invaginated and sunk completely into the yolk, the serosa formed an uninterrupted outer layer to the yolk. The serosa was a very thin cellular layer, and its nuclei were extremely flattened. The first formation of the amnion has already been described (p. 83). It was made of blastoderm which was pulled in with the germ-band when blastokinesis occurred. When the head sank into the yolk, the amnion was completed, but it remained connected with the serosa for some time (Text-figs. 8 and 10). The germ-band later lay free inside the yolk with the amnion covering its ventral side. All connexion with the surface was lost.

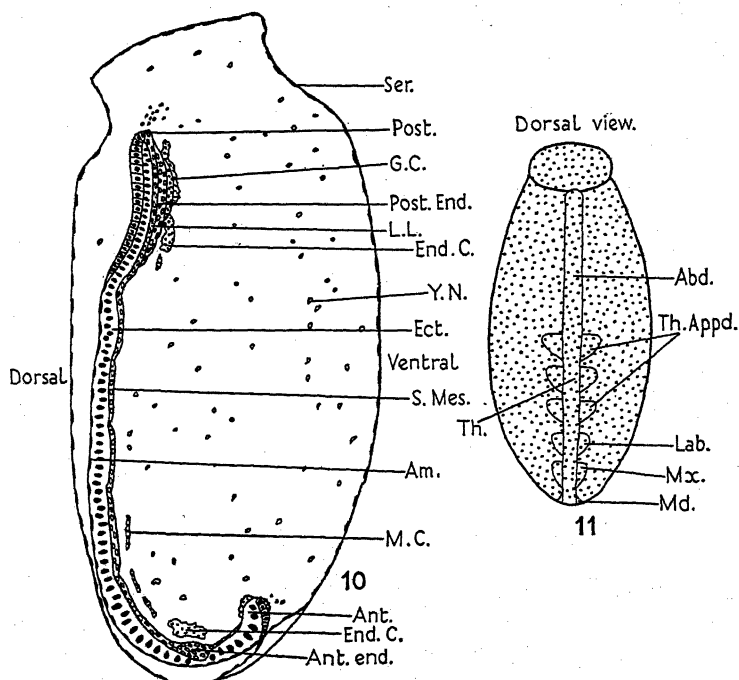
### Formation of the Lower Layer.

Mesoderm in insects may arise in three ways (Imms, 1925): (i) by invagination of the central part of the embryonic rudiment to form a tube; (ii) by overgrowth of the central part by two lateral portions; or (iii) by the proliferation of cells from the ventral plate along the mid-ventral line. In *Rhodnius* the mesoderm arises by a combination of the first two methods.

While the germ-band was in its superficial position (Text-fig. 7), the anterior part of the embryonic plate had become divided into a middle and two lateral plates (Text-fig. 6 d). From the middle plate the lower layer was differentiated by the formation of a groove or gastral furrow which developed in the mid-line (Text-fig. 8 c); this was overgrown by the lateral portions of the ventral plate, the gastral groove being obliterated by the process of overgrowth (Text-fig. 8 B, c, and d). This process took place immediately after the first stage in blastokinesis was completed.

The gastral groove first began just behind the anterior end of the embryo and proceeded from before backwards. A single longitudinal section gave all stages in lower layer formation, from the completed lower layer at the anterior end to the differentiation of the middle plate at the posterior end. During this process cells from the middle plate were given off into the yolk all along the germ-band (Text-fig. 8 A).

After the lower layer was completed, the germ-band became surrounded at its head-end by the amnion, the amnion and



TEXT-FIG. 10. Diagrammatic sagittal section at time of endoderm formation after 96 hours' incubation.  $\times 67$ . *Am.*, amnion; *Ant.*, anterior end of embryo; *Ant. end.*, anterior endoderm rudiment; *End. C.*, 'endoderm' cells degenerating in yolk; *G.C.*, germ-cells; *L.L.*, lower layer; *M.C.*, lower layer-cells degenerating in yolk; *Post.*, posterior end of embryo; *Post. End.*, posterior endoderm rudiment; *S. Mes.*, segmental mesoderm; *Y.N.*, yolk-nucleus.

TEXT-FIG. 11. Diagrammatic drawing of whole mount after 115 hours' incubation.  $\times 45$ . Dorsal view. *Abd.*, abdomen; *Lab.*, labium; *Md.*, mandible; *Mx.*, maxilla; *Th. Appd.*, thoracic appendages; *Th.*, thorax.

serosa having been continuous previous to this stage (Text-fig. 8). The growth of the amnion and the serosa pulled the flexed head end of the embryo into a more superficial position at the

hind-end of the egg (Text-fig. 10). The embryo was now similar to that of *Pyrrhocoris apterus* (Seidel, 1924), except that it was dorsally situated in the yolk, had no caudal flexure, and the germ-cells were already differentiated.

### Formation of the Endoderm.

The formation of the endoderm in insects has been the subject of much controversy, and the various interpretations of its origin have been summarized by Eastham (1930). In *Rhodnius* the endoderm was formed by proliferation from both an anterior and a posterior area of the lower layer. This is somewhat similar to the mode of origin of the endoderm in a number of other insects, for instance *Apis* (Nelson, 1915), *Calliphora* (Noack, 1901), and *Pieris* (Eastham, 1927). In *Pyrrhocoris* Seidel found that the mid-gut was formed from proliferating areas at either end of the lower layer. In *Rhodnius* the anterior endoderm rudiment arose slightly earlier than the posterior. Its position was in the centre of the region of the cervical flexure. Here active proliferation of the cells in the mid-ventral line resulted in a complete disturbance of the regular arrangement of cells of the ventral plate (Text-fig. 10). Many of the cells of the proliferation were given off into the yolk. The others spread out over the ventral surface of the embryo. The cervical flexure made the anterior endoderm rudiment difficult to study in transverse sections.

The posterior endoderm rudiment arose slightly later, near the extreme end of the embryo (Text-fig. 10). It was made by a proliferation of the lower layer, which took place between the lower layer and the germ-cells, but extended farther towards the anterior end of the embryo than did the germ-cells. A large number of these proliferated cells were also given off into the yolk. This posterior rudiment was convenient for examination in transverse sections, there being no caudal flexure in *Rhodnius*.

In a recent paper by Mansour (1934) further evidence is brought forward in support of the view that the mid-gut has an ectodermal origin in some insects. This paper deals with the development of the adult mid-gut in a large number of

Coleoptera, and it is stated that here the adult mid-gut is developed from ectodermal cells of the larva. Mansour claims that this supports his view regarding the embryological ectodermal origin of the mid-gut in the beetle *Calandra* (Mansour, 1927).

At the time when the endodermal proliferations were formed in *Rhodnius* there were no stomodaeal or proctodaeal invaginations. The fate of the endodermal proliferations, and their connexion with the ectoderm of the fore- and hind-gut, will be discussed when the organogeny of *Rhodnius* is described.

About the time that the endoderm began to be formed the head and thorax underwent segmentation, the lower layer becoming constricted between the segments. This segmentation was visible in whole mounts of the embryo (Text-fig. 9 B). The abdominal region remained quite unsegmented at this stage. Certain of the ectodermal cells on either side of the mid-line had become differentiated by this time into specially large conspicuous cells. These were the neuroblast cells, which later formed the ventral nerve-cord (fig. 9, Pl. 5). A few hours later the paired appendages developed (Text-fig. 11).

The cells which were given off into the yolk from the endoderm gradually disintegrated, like those of the mesoderm (see p. 84 above). Presumably their function was to render the yolk substance more easily assimilable. The amount of yolk in *Rhodnius* is very large compared with the amount of embryonic tissue, and this may account for the very large number of embryonic cells given off into the yolk during all stages of the early development.

In conclusion I should like to thank Professor D. M. S. Watson (in whose department this work was done) and Professor L. E. S. Eastham for reading the manuscript of this paper and for giving me many helpful suggestions.

#### SUMMARY.

1. Eggs of *Rhodnius prolixus* were incubated at constant temperature and humidity (21° C. and 90 per cent. relative humidity). Eighty-five per cent. was the lowest record of the controls hatched successfully under these conditions.

2. The processes of maturation and fertilization were not studied.

3. Cleavage begins 12–13 hours after incubation. At 25 hours there are 32 nuclei. Yolk-cells are derived from cleavage nuclei, and they multiply by mitosis up to 50 hours. Blastoderm formation is complete after 55–60 hours of incubation.

4. The ventral embryonic rudiment is similar to that of many other insects. As soon as it is formed, germ-cells are budded off at the posterior pole of the egg.

5. The first stage in blastokinesis is fully described.

6. The formation of the mesoderm is by invagination and overgrowth.

7. The endoderm arises from two proliferating areas situated anteriorly and posteriorly.

8. Numerous cells are given off into the yolk during the early development of the embryo. There they disintegrate.

TABLE 1.

*Rhodnius prolixus*: time-table of early development.

<i>No. of Hours after being Laid.</i>	<i>Stage reached in Development.</i>
$\frac{1}{2}$	Usually a single nucleus in centre of egg. The egg nucleus.
2	Maturation begun.
2–11	Maturation and fertilization.
11–14	First cleavage.
18	Between four and eight cleavage nuclei.
24	Thirty-two cleavage nuclei. Migration to periphery begun.
30–50	Formation of syncytial layer round yolk. Yolk-nuclei increase by mitosis.
50–60	Blastoderm formation—differentiating into embryonic and extra-embryonic blastoderm.
60–76	Ventral embryonic rudiment appeared—germ-cells budded off at posterior end of blastoderm.
76–86	Ventro-lateral portion of embryonic blastoderm withdrawn ventrally. Invagination and involution of embryonic rudiment.
86–106	Formation of lower layer—growth of amnion folds to cover head-end. Anterior and posterior endoderm proliferating begun—segmentation of head and thorax.
108–20	Appearance of head and thoracic appendages. Abdomen quite unsegmented.

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## DESCRIPTION OF PLATE 5.

## ABBREVIATIONS USED FOR PLATE.

*Am.*, amnion; *Bl.*, blastoderm; *C.N.*, cleavage nuclei; *Cy.*, cytoplasm; *Div.nuc.*, nucleus undergoing mitosis; *Ect.*, ectoderm; *E.R.*, embryonic rudiment; *Ex.E.Bl.*, extra-embryonic blastoderm; *G.C.*, germ-cells; *L.L.*, lower layer; *Neur.*, neuroblast cell; *Nuc.*, nucleus; *P.C.*, peripheral layer of cytoplasm; *Ser.*, serosa; *S.Y.*, space in yolk; *Y.*, yolk; *Y.N.*, yolk-nucleus (vitellophage).

Fig. 1.—Cleavage nuclei at 16-32 stage after 24 hours of incubation.  $\times 375$ .

Fig. 2.—Early blastoderm formation. Cleavage nuclei have reached peripheral layer of cytoplasm. One nucleus shows tangential division.  $\times 930$ .

Fig. 3.—Blastoderm formation just later than Fig. 2.  $\times 930$ .

Fig. 4.—Syncytial layer after 50 hours of incubation, in region which will form embryonic blastoderm.  $\times 930$ .

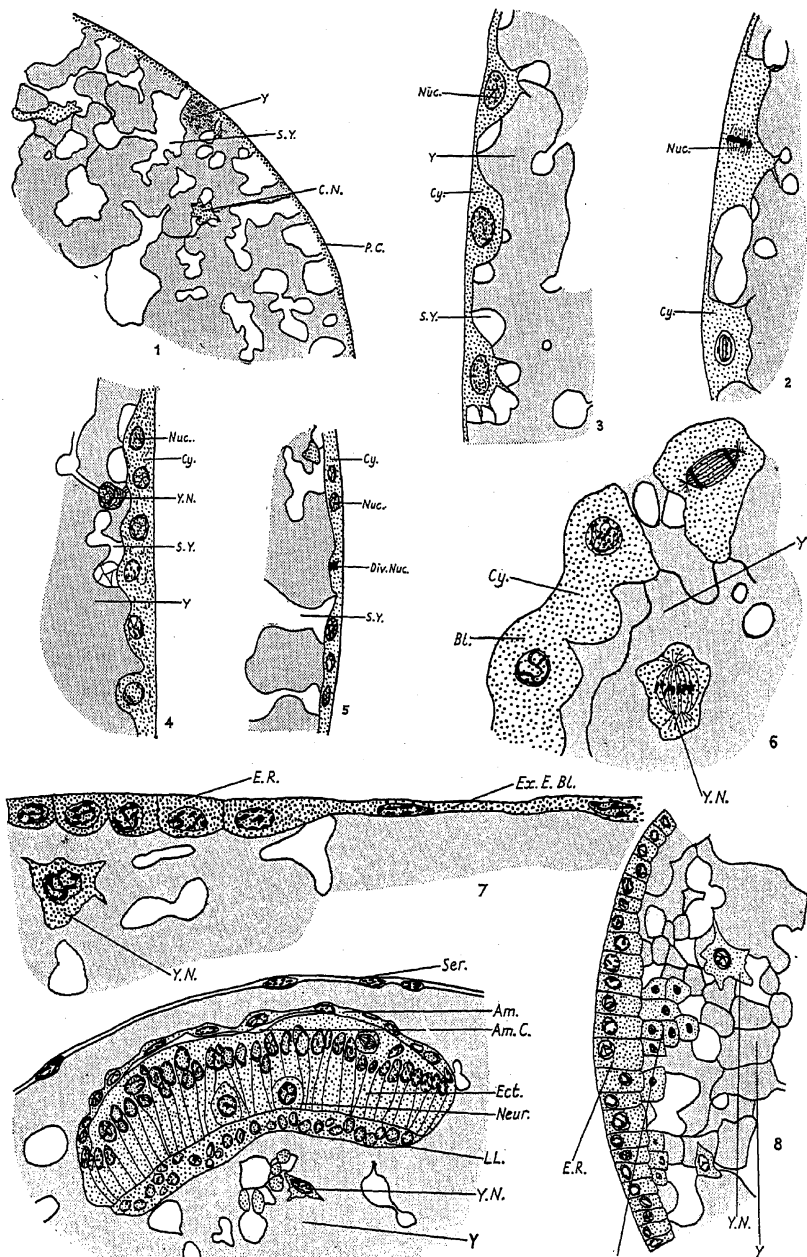
Fig. 5.—Syncytial layer after 50 hours of incubation, in region which will form extra-embryonic blastoderm.  $\times 930$ .

Fig. 6.—Syncytial layer after 50 hours of incubation, posterior end of egg.  $\times 620$ .

Fig. 7.—Junction of embryonic rudiment with extra-embryonic blastoderm.  $\times 620$ . After 76 hours of incubation.

Fig. 8.—Longitudinal section. Embryonic rudiment posterior pole showing origin of germ-cells after 76 hours of incubation.  $\times 620$ .

Fig. 9.—Transverse section embryo. Thoracic region after about 110 hours of incubation.  $\times 250$ . Shows the neuroblast cells.







# Observations on the Embryology of *Corynodes pusis* (Coleoptera, Chrysomelidae).

By

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With Plates 6 and 7.

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A STUDY of the embryology of *Corynodes pusis* Marsh was decided upon in order that its development might be compared with that of *Euryope terminalis* Baly, which the writer had recently (1931, 1932) dealt with in considerable detail. Several features in the ontogeny of the latter species, including the maturation divisions of the female pronucleus and the method of formation of the mesenteron, had proved of interest, and it was felt desirable that further observations be carried out on some related species in order to determine if these phases were merely isolated developmental occurrences peculiar to *Euryope terminalis*, or if they would be recognized in similar stages of any other species of the Chrysomelidae.

Considerable attention has from time to time been directed towards the embryology of various species of Coleoptera, while the results of several of the investigations on certain of the Chrysomelidae are now more or less recognized as classical works on the subject of Coleopterous embryology. Among the most notable accounts are those of Graber (1888-90) on *Lina tremulae*, Wheeler (1889) on *Doryphora decemlineata*, Lécuillon (1898) on *Glytra laeviuscula*, Gastrophysa raphani, *Chrysomela menthastri*, *Lina populi*, *Lina tremulae*, and *Agelastica alni*, Strindberg (1913) on *Chrysomela hyperici*, and of Hirschler (1924-7) on *Donacia crassipes*. The observations of these authors tend to show that on the whole there is considerable agreement as to the main features of the embryology of the Chrysomelidae, but in spite of their carefully considered conclusions there is still a certain amount of doubt in regard to certain points, and

especially those which for years have proved such controversial questions in the subject of insect embryology as a whole. Thus we find that while Lécaillon, who made a detailed study of as many as six different species, considers that the mesenteron is ectodermal in origin in the Chrysomelidae, the other authors maintain that its wall is derived from endodermal cells. All of them are more or less decided on a bipolar origin for the rudiment of the mid-gut wall, while Strindberg (1913) and Hirschler (1924-7) find a vestige of a median endoderm in addition to anterior and posterior rudiments. The derivation of the mesenteron from two rudiments at the blind ends of the stomodaeum and proctodaeum is in accordance with the findings of the majority of recent workers in other groups of insects (Nelson, 1915; Seidel, 1924; Mansour, 1927; Eastham, 1927, 1930*a* and *b*; Henson, 1932), and this now seems to be the most generally accepted view.

The conclusions of Leuzinger and Wiesmann (1926), whose article on the embryology of *Carausius morosus* is one of the outstanding contributions to the subject in recent years, form a notable exception to the general opinion of to-day. As a result of a very thorough and comprehensive examination of this Orthopteron these authors conclude that there are slight ectodermal extensions of the wall of the proctodaeum, but state very definitely that they have nothing to do with the formation of the mid-gut. Their observations seem to prove that in the Orthoptera at least there is no bipolarity of the mesenteron rudiment, but that it is formed from secondary endoderm derived from the 'Blutzellenlamelle', which Eastham (1930*a* and *b*) considers to correspond to the median mesoderm of *Pieris*.

The present writer has also failed to find any anterior or posterior rudiments of the mid-gut in *Euryope* (1932), the wall of which was thought to be derived from a median longitudinal layer of endoderm similar to that observed by Strindberg (1913) and Hirschler (1924-7) in other species of Chrysomelidae, but to which little importance was attached. There is no doubt that this median layer of endoderm in the Chrysomelidae corresponds to the median mesoderm of *Pieris* and to the Blutzellenlamelle described by Leuzinger and Wiesmann

in *Carausius*. The findings of the latter two workers are, therefore, of interest and importance in view of the fact that the layer which gives rise to the mesenteron wall in *Carausius* is identical with the one in *Euryope* and other Chrysomelid embryos.

Although this question has perhaps been somewhat over-emphasized and laboured in regard to insect embryology in general, it was with a view to substantiating these observations on *Euryope* that the embryology of *Corynodes* was undertaken. In addition, certain other features of the embryology are treated in some detail in view of some interesting contributions which have recently been published.

*Corynodes pusis* was selected for investigation and for comparison with *Euryope* as both species, in addition to being closely related in their adult characters, have approximately the same embryonic period and their first stage larvae are strikingly similar in appearance. Further, they have the same food-plant, *Asclepias fruticosa*, which is of common occurrence along the Witwatersrand. Occasionally the two species were found together in nature, and while both seem to be somewhat spasmodic in their distribution, occurring in somewhat isolated localities, it was noticed that, on the whole, *Corynodes* was found in relatively greater numbers than *Euryope*, but that the latter species seems at present to be rather more widely distributed. *Euryope* was collected in Johannesburg and on the West Rand as far as Krugersdorp and the Hekpoort district, whereas so far *Corynodes* has not been found by the writer in Johannesburg, although it occurs in the other areas. Both species were probably formerly much more widely distributed than they are at present, but for reasons such as building and mining operations they have been disturbed, and their food-plant, although still fairly common, is perhaps also not quite so plentiful.

The expectation that a comparison of the embryology of these two closely related species would yield interesting and instructive results has been more or less fully realized, for, as the present paper indicates, the writer is forced to almost the same conclusions in interpreting the development of *Corynodes*

as were expressed for that of *Euryope*. The main differences lie in their relative rates of development, but these slight variations do not materially interfere with the comparison of even the more detailed and finer points in their embryology.

#### MATERIAL AND METHODS.

Adult beetles were collected in bright sunshine during December at Krugersdorp and Hekpoort, at both of which localities they were numerous on *Asclepias fruticosa*. They are much more active fliers than *Euryope terminalis*; but, like that species and many other Chrysomelidae, they simulate death when approached, and many escape by simply dropping to the ground.

Many of the beetles were copulating at the times of collection, and oviposition took place freely in the laboratory during the latter half of December and the first week in January. Like *Euryope*, these beetles were easily kept in the laboratory in large museum jars and supplied daily with fresh food. Confinement in the jars did not in any way seem to hinder oviposition or decrease their fertility, for numerous eggs were collected each day.

The eggs, which were of a bright yellow colour changing to a duller shade shortly after oviposition, were usually laid in rows of seven to ten or eleven in the curled leaf edges, or between two closely apposed leaves and occasionally in the flowers.

The method of oviposition and protection of the eggs is essentially similar to that of *Euryope terminalis* and other Chrysomelids, but in this case the so-called epichorion forms a much sparser covering than that described in other Chrysomelidae (Lécaillon, 1898; Paterson, 1931). In *Corynodes* it merely serves as a viscous material for attaching the eggs to the leaves, and sometimes for cementing two leaves together, so that the eggs may be laid between them. It was not always observed, for occasionally the females laid the eggs in loose clusters on the stems or leaves and these invariably fell to the bottom of the jar.

As a rule, after the first egg was laid, those following were deposited alternately on opposite sides of the first until the row was completed.

As in Europe, it was found that embryonic life lasted from 18 to 21 days, but examination of the sections later showed that there were slight variations in the rates of development of different eggs. As soon as the eggs were deposited they were set aside and allowed to develop for varying periods, ranging from oviposition until the nineteenth day.

Considerable difficulty was experienced in obtaining satisfactory preparations, as at some stages the yolk seemed to become hard and brittle. Technique similar to that described for Europe (1931) was employed, but on account of the brittleness of the yolk, the eggs were allowed to remain for as long as a week in celloidin before infiltrating with paraffin wax. As an alternative to the alcoholic Bouin fixative used for Europe, a mixture of 3 per cent. nitric acid and corrosive sublimate, as recommended by Leuzinger (1926), was also tried, but with less satisfactory results.

Apathy's method (Guyer, 1917) was employed for some of the more brittle stages. This method takes considerably longer than that of Newth (1919), as it requires a more gradual infiltration of celloidin, the block of which is subsequently hardened in chloroform and cleared in an oil mixture consisting of four parts each of chloroform, origanum oil, and cedarwood oil, with one part each of absolute alcohol and carbolic acid crystals. It was found that in specimens thus treated the yolk remained intact and the sections were less inclined to break and tear.

Transverse and longitudinal sections of  $5\mu$  and  $6\mu$  thick were cut and stained either in Hansen's or Ehrlich's haematoxylin, eosin being used as the counterstain.

#### THE EARLY STAGES OF DEVELOPMENT.

Relatively few workers on insect embryology have reported on the initial stages of development, and the maturation divisions seem particularly to have been neglected. This is probably due to the fact that many insect eggs when laid have already undergone the preliminary cleavage stages, so that material for the study of the earlier phases would in such cases be more difficult to procure.

Of the accounts of the embryology of the Chrysomelidae,

Wheeler's (1889) is the only one in which there is any description of the maturation divisions, while Hegner (1909, 1911), although not investigating the embryology of the Chrysomelidae, gives some account of certain of the structures of the eggs at oviposition. Lécaillon (1898), while figuring the eggs of several species of this family, has apparently not observed the maturation stages, for he makes no mention of them.

From the two species investigated by the writer it would seem that the eggs of Chrysomelidae are favourable for the study of the maturation divisions of the female pronucleus, for in both *Euryope* and *Corynodes* it was observed that the egg at oviposition was undergoing maturation. A similar condition has also been described in the egg of *Eudemis* by Huie (1917-18), and is probably more general in Lepidoptera than is realized.

The eggs of *Corynodes* are elongate-oval, measuring about 1.25 mm. by 0.5 mm. The structure is essentially similar to that of *Euryope* at the same stage, but, with the exception of one doubtful case, the male and female pronuclei were not observed together in any of the preparations. The central mass of yolk-globules (figs. 1-4, *yk.*, Pl. 6), interspersed by a fine reticulation of cytoplasmic granules, is surrounded by a peripheral periplasm (figs. 1-4, *per.*, Pl. 6), which is a narrower layer than that of *Euryope*. Its cytoplasm is apparently less selective of stains than that of *Euryope*, for the differentiation into inner basophil and outer eosinophil strata was less noticeable. The periplasm is, however, a well-defined layer of finely granular cytoplasm, which equatorially is enlarged into the polar-plasm (fig. 2, *pp.*, Pl. 6) where the maturation divisions of the female pronucleus take place. In *Euryope* the polar-plasm was located in the anterior half of the egg, and not in the middle region as in *Corynodes*.

At oviposition most of the eggs were found to be in a state of maturation, the first polar spindle (fig. 1, *ps.*, Pl. 6) being observed in the polar-plasm. The spindle is more or less barrel-shaped, its ends are rounded, and it seems to conform to the general characteristics of the anastral type of meiosis described by Wilson (1925). It was usually directed perpendicularly to

the periphery, but in one exceptional case, in which the spindle was rather small, the longitudinal axis of the spindle was parallel to that of the egg.

At least six deeply staining basophil chromosomes (fig. 1, *cr.*, Pl. 6) were observed at each extremity of the spindle, the outer pole of which was situated in the eosinophil layer of the polar-plasm, while the spindle threads could be traced in the basophil layer, with the inner pole lying near the boundary of the yolk and the periplasm.

Huie (1917-18) has observed that in *Eudemis* the first polar body, after extrusion from the polar-plasm, undergoes the usual division into two equal daughter cells.

In *Corynodes*, coincident with the formation of the second polar spindle at the tenth hour, there is also some indication that the first polar body is dividing further (fig. 4, Pl. 6). A small spindle (*s.*), the threads of which were rather indistinct and the axis of which was perpendicular to the surface, was observed in the polar-plasm just anterior to the larger spindle (*ps.*<sub>2</sub>). The ultimate division into two polycytes was not observed, but it is assumed that, as in *Eudemis*, two daughter polycytes result from this division before the polar body disintegrates.

Thirty minutes after oviposition the spindle (fig. 2, Pl. 6) appears to be rather more elongate, its outer pole projecting freely into the peripheral cytoplasm and causing a slight protuberance of the egg surface.

The separation of the first polar body from the pronucleus seems to begin shortly after this, and at 2 hours it is extruded from the polar-plasm, and appears as a distinct rounded mass (fig. 3, *pb.*, Pl. 6) lying at the periphery. It consists of fine granules containing twelve to fourteen chromosomes, but no definite enclosing membrane could be distinguished.

At this stage the female pronucleus (fig. 3, *pn.*, Pl. 6) has sunk slightly into the yolk, but it still retains a connexion with the polar-plasm by means of a fine network of cytoplasmic threads. As in the first polar body, fourteen chromosomes were counted in the pronucleus, which is an almost regular spherical mass, also devoid of a nuclear membrane.



The chromosomes of the first polar body gradually become less distinct in later stages, while the female pronucleus again migrates to the periphery, the second polar division occurring 10 hours after oviposition (fig. 4, Pl. 6). This division takes place rather later than it does in *Euryope*, where it occurred at the sixth hour, and it also differs in that the spindle is much smaller and is not parallel, but perpendicular, to the surface. Wheeler (1889) has made similar observations regarding the polar spindles of *Blatta*, in which the axes of both spindles are perpendicular to the surface.

The actual process of conjugation of the male and female pronuclei was not observed in any of the preparations of *Corynodes*, but, as in *Euryope*, the first cleavage division of the zygote nucleus occurs shortly after the extrusion of the second polar body. Subsequent divisions take place rapidly, so that by the fifteenth hour after oviposition there are numerous cleavage nuclei scattered throughout the yolk, with a slight preponderance of numbers in the anterior half. At this stage the remains of the second polar body may still be recognized outside the periplasm.

The majority of the cleavage nuclei are destined to form the blastoderm layer at the surface, but some of them lag behind in the yolk, constituting the so-called vitellophags or yolk-nuclei (figs. 8 and 17, *vt.*, Pl. 6). The method of blastoderm formation agrees in all essentials with that described in *Euryope* (1931), the cleavage nuclei travelling through the yolk and arranging themselves parallel to the surface, those in the anterior half appearing to reach the periplasm more quickly than those in the posterior half. Eventually numbers of nuclei enter the periplasm to form a surface layer of uniform thickness, which, immediately following on the entrance of the nuclei into it, has a crenated appearance similar to that observed in *Euryope*.

Shortly after their entrance into the periplasm the nuclei increase by further divisions, the axes of the spindles being parallel to the surface. Cell-walls and basement membranes are next formed, and by about the forty-eighth hour the formation of the blastoderm is complete.

Simultaneously with the completion of the blastoderm the

genital rudiment (fig. 5, *gr.*, Pl. 6) is differentiated at the posterior extremity of the egg. Previously to this, i.e. at oviposition and during the outward migration of the cleavage nuclei, there occurs an elongate granular mass (fig. 6, *gp.*, Pl. 6) at the posterior pole. This structure is quite distinct from the periplasm, than which it stains more deeply. A similar, but rather broader structure, was also seen in the early stages of *Euryope*, and is probably characteristic of the majority of Chrysomelidae. It is thought to constitute a germinal substance, and has been termed the germinal protoplasm or 'pole disc' by Hegner (1909, 1911). Certain of the cleavage nuclei after passing through this protoplasm at the time of blastoderm formation congregate at the posterior pole, forming a conspicuous mass of cells (fig. 5, *gr.*, Pl. 6), the genital rudiment, from which the paired gonads later develop.

The derivation of the vitellophags or trophocytes, as Hagan (1931) prefers to call them, from the cleavage nuclei before the blastoderm is formed, agrees with the observations on *Euryope*, and, although no great significance is attached thereto, it may indicate that this is their usual method of formation in the Chrysomelidae at least, if not in the majority of Coleoptera.

During the formation of the embryonic rudiment, the vitellophags undergo divisions which also seem to be anastral. Just prior to the gastrulation phases they were observed in clusters or rings of three to eight or more nuclei (fig. 17, *vt.*, Pl. 7), and soon after this, almost synchronizing with gastrulation, the yolk becomes marked off into polyhedral areas, each of which may contain one or more nuclei. This condition, which represents the secondary cleavage of the yolk, was hardly distinguishable in *Euryope*, but it is characteristic of all the post-gastrulation stages of *Corynodes*, and is apparently of fairly general occurrence in Orthopterous, Lepidopterous, and Coleopterous eggs (Imms, 1925).

At the close of this stage, which probably represents the blastula of other embryos, the egg consists of a single layer of cells, the blastoderm, enclosing a central mass of yolk-cells. A slight dorsal cytoplasmic thickening, containing a few scattered nuclei, was observed in the anterior region. This seems

to be comparable to the primary dorsal organ described by Strindberg (1913) in *Chrysomela* and by Hirschler (1924-7) in *Donacia*, while a similar transitory structure was also observed in *Euryope*. Lemoine (1883) has also reported on an anterior dorsal thickening under the blastoderm in *Anurophorus*, and considered that it probably represented the dorsal organ of Crustacean eggs and was analogous to the cumulus of an Arachnid, from which it differed, however, in persisting throughout the whole embryonic period. According to MacBride (1914) the primitive cumulus of an Arachnid egg corresponds to the genital rudiment of an insect egg, which is usually postero-ventral in position. This dorsal thickening in the Chrysomelidae is extremely short-lived, and no special significance can be attached to it. It certainly has no connexion with the formation of the genital cells.

#### FORMATION OF THE EMBRYO.

As in the majority of insects, the embryonic rudiment of *Corynodes* (fig. 8, *ex.*, Pl. 6) develops as a differentiation of the blastoderm cells along the whole length of the ventral surface. At first it is differentiated only in the middle third, and immediately upon its appearance the yolk adjoining it forms a sharply defined layer, which appears to be more granular than the rest and probably represents a concentration of cytoplasm along the dorsal wall of the embryonic rudiment. The rudiment takes the form of a band of somewhat closely arranged columnar cells, which are readily distinguished from the flatter widely separated cells of the extra-embryonic blastoderm (fig. 8, *ex.*, Pl. 6), as the undifferentiated blastoderm is now termed.

In its early stages the embryonic rudiment is confined to the ventral surface, but later the growth of the body results in a certain amount of change in position. At the end of the gastrulation period and during the formation of the appendages, the embryo elongates rapidly and its posterior extremity curves right round on to the dorsal surface. On the sixth day, when the appendages are established as small lobular processes, this caudal flexure reaches about half-way along the dorsal surface

and ends in a very conspicuous mass, which is more deeply embedded in the yolk than the rest of the embryo. In *Corynodes* the cephalic extremity is never involved in any marked blastokinesis. It always remains antero-ventral in position in its early stages, and it is only during and after the dorsal closure of the head that some of its parts extend on to the dorsal surface.

On the eighth day, by which time the appendages are conspicuous, the abdominal region seems to diminish; its segments come to lie closer together, with the result that there is no longer any dorsal caudal flexure, the embryo lying straight along the ventral surface and extending from anterior to posterior pole. As a result of this straightening of the embryo, the anus, which prior to this appeared as an invagination in the conspicuous caudal extremity, is carried from the dorsal surface into a posterior more ventral position. Although the cephalic end of the embryo remains more or less stationary, the mouth undergoes a slight change in position, which coincides with the blastokinesis of the abdominal region. The stomodaeum originates as an ectodermal invagination behind the labral segment almost simultaneously with the appendages. The mouth is, therefore, at first anterior and almost terminal in position, but when the protocerebral lobes develop and expand dorso-laterally, and the antennae migrate from a post-oral into a pre-oral position, the mouth shifts backwards and downwards on to the ventral surface. Similar movements were observed in the embryo of *Euryope*, but in that species there is a slight dorsal flexure of the cephalic end, which, however, reverts to the ventral surface.

The process of gastrulation is similar to that of *Euryope*, and, with the exception of *Gastrophysa* (Lécaillon, 1898), the *Chrysomelidae* which, so far, have been investigated (Strindberg, 1913; Hirschler, 1924-7) seem to present a remarkable constancy in regard to this and several other important developmental features.

Along the mid-ventral line of the embryonic rudiment an area of active cell-division becomes evident on the third day. On the following day this middle plate of cells (figs. 9, Pl. 6,

and 19, *mp.*, Pl. 7) is invaginated dorsally into the yolk, the process being that of gastrulation by invagination. The gastral groove (figs. 9 and 19, *gg.*) appears at first as a shallow depression, which deepens into a narrow slit (fig. 13, *gg.*, Pl. 6) and eventually closes. In this way the cells of the middle plate are invaginated into the yolk to form the lower layer of cells (fig. 30, *ll.*, Pl. 7). This process takes place rapidly, beginning in the middle, then in the posterior, and finally in the anterior region, so that while the gastral groove is still a somewhat shallow furrow in the anterior third of the embryo, it has already closed and the lower layer is evident in the posterior two-thirds.

#### THE EMBRYONIC MEMBRANES.

In Euryope it was observed that, concurrently with the differentiation of the middle and lateral plates, the amniotic folds developed from the extra-embryonic blastoderm at the two extremities of the embryo. In *Corynodes* the amniotic folds (fig. 30, *af.*, Pl. 7) appear rather later, arising simultaneously during the process of gastrulation. They grow towards each other over the ventral surface of the embryo, but have not yet united in the equatorial region when the gastrulation processes have been completed there (fig. 30, Pl. 7).

As a result of the fusion of the head and tail folds, the amnion (figs. 13, Pl. 6, 22, and 30, *am.*, Pl. 7) becomes separated from the serosa (figs. 13 and 30, *ser.*). The latter is a uniform layer of cells, which during later embryonic life become gradually less columnar in appearance. After the separation of the two embryonic membranes the anterior part of the embryo sinks slightly into the yolk, and again a finely granular cytoplasmic layer, similar to the one observed in contact with the embryonic rudiment, is differentiated from the yolk.

The amnion is at first composed of well-defined, more or less, columnar cells, but it soon becomes stretched over the ventral surface of the growing embryo as an extremely thin layer, the elongate-oval nuclei of which alone are usually discernible.

After the appendages have been established and during the forward migration of the antennae, the amnion ruptures mid-

ventrally, and, growing dorso-laterally, eventually extends over the dorsal surface, enclosing the yolk (fig. 7, *am.*, Pl. 6).

The ultimate fate of the amnion was not traced in *Corynodes*, but, following the works of Graber (1888-90), Wheeler (1889), and Hirschler (1924-7), it is now generally accepted that in the majority of Chrysomelidae, when the embryo closes dorsally, the remains of the amnion are withdrawn into the yolk where they disintegrate.

The serosa (figs. 13, Pl. 6, and 30, *ser.*, Pl. 7) in both *Euryope* and *Corynodes* is retained throughout embryonic life as a limiting layer underlying the vitelline membrane (figs. 13 and 30, *vm.*), and it can still be recognized just previous to hatching.

#### THE GERM-LAYERS.

From Eastham's recent summary (1930*b*) of the various opinions regarding the formation and development of the embryonic layers, it is evident that, in spite of the enormous amount of work on the subject, no general uniformity of interpretation has, as yet, been arrived at.

The opinion that the stage at the close of the blastoderm formation represents a blastula seems to be generally accepted, but whether the invagination processes are homologous with the gastrulation of other invertebrates is still a controversial subject. Eastham and others are of the opinion that it is a secondary feature, while the present writer is inclined to agree with Mansour (1927) that the process represents gastrulation. It is true that it does not fulfil all the conditions of a normal embolic gastrula, but, nevertheless, as a result of the invagination of the middle plate-cells, the three germinal layers of the embryo are established. Eastham (1930*b*), while agreeing that it is probable that this process of invagination may represent gastrulation, is of the opinion that it is so divergent from the normal process of gastrula formation that it should be regarded as a secondary feature peculiar to the Insecta. His chief objection to interpreting the ventral furrow as a gastral groove is the variability in the time and method of formation of the mesoderm and endoderm throughout the various orders of insects. Criticizing Mansour's (1927) definition of a gastrula, and,

in view of the fact that the majority of present-day investigators accept the bipolarity of the endoderm rudiment, he decides that 'it is plain that any invagination which occurs in the insect germ-band is more concerned with mesoderm than endoderm, since the latter (if present at all) is only found anteriorly and posteriorly'.

In *Euryope* and *Corynodes* I have failed to find any indication of anterior or posterior endoderm rudiments; for the lower layer of cells which results from invagination gives rise to both mesoderm and endoderm in all the body segments and certain of the head segments. Mansour's definition of a gastrula is, therefore, partly applicable to these two embryos, for some of the cells which are invaginated do give rise to the lining of the mesenteron, and these cells are considered to be endodermal. This embryonic stage in the development of *Euryope* and *Corynodes* is for this reason considered to be comparable with an embolic gastrula, its divergence from the normal being probably due to the large central mass of yolk.

In both species which have been investigated by the writer, after the completion of gastrulation, the embryo is composed of two well-defined layers of cells—a ventral layer, the ectoderm (fig. 30, *ect.*, Pl. 7), over the outer surface of which stretches the amnion, and a dorsal layer, the so-called lower layer of cells (fig. 30, *ll.*, Pl. 7), which abuts on the central yolk-mass. Differentiation of the cells of the lower layer takes place very rapidly, and on the day following the completion of the gastrulation processes (i.e. the fifth day) it is marked off into a pair of lateral masses, which are joined by a median layer. The former are two cells in thickness and are regarded as the mesoderm (fig. 22, *ms.*, Pl. 7), while the latter, consisting of a single layer stretching over the developing nerve-cord, is considered to be the rudiment of the endoderm (fig. 22, *end.*, Pl. 7).

The three embryonic layers have now been established, and further development involves the gradual formation of the various organs and structures which function in the first larval instar.

#### LATER DEVELOPMENT OF THE EMBRYO.

Shortly after the differentiation of the lower layer of cells, the anterior extremity of the embryo begins to expand laterally,

and at an early stage the large procephalic lobes become evident. The region posterior to this is about half as wide, is somewhat elongate and strap-shaped, and soon becomes marked off externally into a series of well-defined segments, those in the anterior region, comprising the head and thorax, being shortly recognized by the development of appendages in addition to other criteria. The abdominal segments are devoid of appendages in this species, as well as in *Euryope*, although they may occur in other Coleopterous embryos, including the Chrysomelid, *Doryphora*, in which Wheeler (1889) observed appendicular structures on the first abdominal segment. The embryo of *Corynodes*, in agreement with that of *Euryope* and many other typical insect embryos, consists of six cephalic, three thoracic, and eleven abdominal segments.

All the appendages are paired and are formed in a uniform manner as small hollow outgrowths of the ectodermal wall, lateral to the developing nerve-cord. As development proceeds the appendages elongate and become more pronounced, those of the thoracic segments being particularly conspicuous, and extending for at least two segments behind that on which they arise. As in *Euryope*, the thoracic legs become slightly tripartite in appearance, foreshadowing the coxal, femoral, and tibial joints of the larval leg, the trochanter and tarsus of which have not yet been differentiated.

A short distance in front of the stomodaeal invagination the labrum (fig. 31, *lr.*, Pl. 7) arises on the large procephalic segment as a pair of fairly conspicuous ventral lobes, which are directly continuous at their lateral margins with the protocerebral lobes of the brain (*pc.*), the nervous system developing synchronously with the appendages. Associated with these labral structures is the labral mesoderm, and there can be no doubt that there is at least one pre-oral segment in these and other insect embryos. Eastham (1930 *a*) has carefully investigated the condition in the embryo of *Pieris*, and also describes a primary bilobed origin for the labrum. In addition, he finds that the epipharynx is also slightly bilobed, but, after considering the development of the pre-oral mesoderm and neuromeres, he concludes that the small epipharyngeal lobes cannot be regarded



as indications of a second pre-oral metamere, the head of *Pieris* being, therefore, composed of six segments. It is of interest to note that Wiesmann (1926) finds evidence in *Carausius* of a pre-antennary segment, which is indicated by the development of rudimentary coelomic cavities and rudimentary appendages. The head of *Carausius* consists, therefore, of seven segments, as does that of *Doryphora*, according to Wheeler (1889). In *Anurida* Denis (1927) also finds that the head is composed of seven metameres, in addition to an anterior 'région acronale'. There seems, however, to be no embryological evidence for the pre-antennular segment of Denis in *Anurida*, its presence being suggested by the condition of the neuromere and the mesoderm.

In *Corynodes* there are no traces of pre-antennary appendages, nor do the mesoblastic somites indicate the presence of such a segment. The epipharynx does not appear to be separated off primarily as a bilobed structure, but seems from the beginning to be an undivided region immediately posterior to the labrum, but having no segmental significance.

Post-orally there develop four pairs of metamERICALLY arranged appendages, the antennae (fig. 20, *at.*, Pl. 7), mandibles (fig. 21, *md.*, Pl. 7), first (fig. 10, *mx.*, Pl. 6) and second maxillae. The antennae (fig. 32, *at.*, Pl. 7), which are never so strongly developed as the mandibles or maxillae, are primarily post-oral in position. They originate as small protuberances near the posterior border of the procephalic lobes, but with the subsequent rapid growth of the anterior and lateral cephalic structures, during which the cerebral ganglia become greatly enlarged and the labral segment and mouth move backwards, the antennae eventually assume a pre-oral position. In *Corynodes* the appendages appear on the fifth day; by the seventh day the forward migration of the antennae may be observed; and on the ninth day the mouth is ventral, while the antennae are dorso-laterally placed pre-orally.

Between the antennary and mandibular segments in the young embryo there is a well-defined segment, the intercalary or pre-mandibular, which only persists while the antennae are post-oral in position. It has a pair of fairly conspicuous mesoblastic

somites with doubtful cavities, and although the outer wall presents a pair of slight lateral undulations, somewhat similar to those of *Pieris*, it could not definitely be decided whether these represented appendages or not. There is, however, no doubt as to the presence of the segment, which occurs at that part where the embryo narrows considerably behind the antennary segment.

The remaining three cephalic segments bear conspicuous paired appendages, those on the mandibular and maxillary segments being better developed than the labial (figs. 11 and 12, *lp.*, Pl. 6), but all three pairs arise similarly as lobular protuberances of the ventro-lateral ectoderm.

The distance between the labial appendages and the base of the first thoracic leg is considerable, and seems to indicate that there is a large intersegmental region between the head and thorax, and that the prothoracic segment is somewhat larger than the others.

#### FURTHER DEVELOPMENT OF THE ECTODERM.

In addition to giving rise to the metameric appendages, the ectoderm is important in that from it many of the internal organs of the larva are derived by a series of invaginations.

Concurrently with the evagination of the appendages, the mouth appears on the fifth day as a simple ectodermal invagination (fig. 32, *m.*, Pl. 7) at the anterior end of the embryo, immediately behind the labral lobes. As the stomodaeal invagination elongates and advances farther into the yolk in a posterior direction, it becomes apparent that there is only one layer of cells forming its inner wall, and in fairly advanced stages this wall becomes extremely thin, while laterally the walls are comparatively thick. For the greater part of embryonic life the stomodaeum is a relatively straight tube with its long axis parallel to the longitudinal axis of the egg. Towards the close of the embryonic period the crop region becomes differentiated from the oesophagus (fig. 15, *oes.*, Pl. 6) and pharynx (*ph.*) as a rather more dilated portion (fig. 15, *cp.*, Pl. 6), which has a well-marked valve (fig. 15, *v.*, Pl. 6) at its entrance into the mesenteron.

The hind-gut develops from a proctodaeal invagination which

first appears as a very shallow depression on the sixth day when the dorsal caudal flexure is very conspicuous. The invagination is more apparent on the seventh and successive days. Owing to the caudal flexure the proctodaeum is at first directed ventrally, but when the embryo shortens and straightens on the eighth day, the developing proctodaeum is carried into a terminal position, and its axis is then almost parallel to the longitudinal axis of the egg. The elongation of the posterior invagination proceeds more rapidly than the formation of the mid-gut and the absorption of the central yolk, so that, since the distance between the junction of these two parts and the anal aperture (fig. 15, *an.*, Pl. 6) is insufficient to accommodate the whole length of the ingrowing tube, the latter folds on itself and the division of the hind-gut into ileum (fig. 15, *il.*, Pl. 6), colon (*cl.*), and rectum (*rc.*) becomes apparent.

The inner wall of the proctodaeum, like that of the stomodaeum, is composed of a single layer of cells which, as development proceeds, becomes progressively thinner until it eventually forms an extremely fine layer between the lumen of the hind-gut and the yolk-content of the mid-gut.

There is no indication that the cells comprising these thin layers at the inner ends of the stomodaeum and proctodaeum are other than ectodermal. Henson (1932) finds that in *Pieris* these thin layers are composed of endodermal cells which are differentiations of the cells at the blind ends of these two ectodermal regions. In both *Euryope* and *Corynodes* the present writer is of the opinion that the endoderm (figs. 16A, Pl. 7, and 22, *end.*, Pl. 7) is already differentiated before the stomodaeal and proctodaeal invaginations make their appearance, and that, therefore, the thin walls in these regions are merely composed of the greatly stretched ectodermal cells of the original ingrowths. There is no indication, as there is in *Pieris*, that the ectodermal cells differentiate to form a special endodermal partition between the lumina of the three primary sections of the alimentary tract.

#### MALPIGHIAN TUBULES.

Just as the development of the wall of the mid-gut has been a much-discussed subject in insect embryology, the origin and

physiology of the Malpighian tubules have proved almost as puzzling to those who have studied insect anatomy. Some investigators maintain that they arise from the anterior region of the hind-gut and are purely excretory in function, whereas others, like von Gorka (1914), who was chiefly interested in their physiology, attribute them to the mid-gut and consider that they play an important part in digestion. The cells of the Malpighian tubules are very like those of the mid-gut in appearance, and, from their position just at the junction of the mid- and hind-guts, it is naturally somewhat difficult to decide, even after careful histological examination, whether they are outgrowths of the mid-gut as in other Arthropods, or whether they arise from the wall of the hind-gut, in which case the Insecta would differ from all closely related forms.

While this doubt still remains in the minds of the students of insect morphology, the majority of those who have studied the embryology are in agreement that the Malpighian tubules are derived from the wall of the proctodaeum, and are, therefore, composed of ectodermal cells.

Carrière (1890) and Nelson (1915) have found that in *Chalicodoma* and *Apis* respectively the Malpighian tubules are actually formed from the ectoderm before the proctodaeum becomes invaginated. These observations are of interest in view of the fact that in the majority of insect embryos they are later developments, and the general tendency is to regard them as derivatives of the wall of the hind-gut.

Wheeler (1889) found that in *Doryphora* they arose as three pairs of hollow outgrowths of the proctodaeum, shortly after the latter was invaginated. In *Chrysomela* (Strindberg, 1913) they have a similar origin, and Hirschler (1924-7) is also of the opinion that in various insects, including the Chrysomelids *Gastrophysa* and *Donacia*, they should be regarded as diverticula of the proctodaeal wall.

It is, therefore, apparent that students of insect embryology have no doubt about the ectodermal origin of these structures, and this view has been so generally accepted that no particular attention has been focussed on the subject, even though it is always remarked that in the larval stages the cells of the tubules

are not characteristically ectodermal in appearance. Recently, however, Henson (1931, 1932), who after a study of the anatomy of the larva of *Vanessa* was dissatisfied with the acceptance of the ectodermal origin of the Malpighian tubules, has investigated the matter further in the embryo of *Pieris*. He comes to the conclusion that their development is not quite so simple as it would at first appear, each tubule consisting of two distinct portions, each of which is derived from a different set of cell elements. The lower part, which forms the common tube, arises as a simple outgrowth of the proctodaeal wall, but the main portion is formed from an interstitial ring of endodermal cells.

This view marks a departure from the general opinion of embryologists that the whole of the tubule is composed of ectodermal cells, and, if accepted, will probably explain why the cells of the tubules present so marked a resemblance to those of the endodermal wall of the mid-gut. As Henson shows, it would also make it possible to homologize these structures with the tubules in other Arthropods, where there seems to be no question as to their endodermal nature.

In the two Chrysomelid beetles on which the writer has made observations there are three pairs of Malpighian tubules arranged three on each side of the proctodaeum (fig. 16, *Mt.*, Pl. 7). Each tubule, unlike those of *Pieris*, arises separately from the proctodaeum, and there is no common excretory tube or vesicle discharging into the alimentary tract. As is clearly seen in the diagrams (fig. 16 A-E, Pl. 7), they develop one behind the other, the intervals between being about  $6\mu$ . They occur just at the junction of the mid- and hind-guts, but are clearly outgrowths of the proctodaeum (*proc.*), their walls and lumina being continuous with those of that portion of the alimentary tract. Their first origin is shown in figs. 14 A and 14 B, Pl. 6. There is no indication, whatsoever, of an interstitial ring of endodermal cells, which is apparently a marked feature of the Lepidoptera (Ito, 1921; Henson, 1932). The endoderm in *Corynodes* and *Euryope* is an early differentiation of the lower layer, and is a clearly defined, single-layered zone on the dorsal side of the embryo adjacent to the yolk (fig. 16, *end.*, Pl. 7).

The writer therefore concludes that, in the absence of this interstitial ring, and as the tubules are independent evaginations of the proctodaeum, the whole of each tubule in *Corynodes* and *Euryope* is composed of ectodermal cells, which later become surrounded by mesoderm continuous with that on the outer surface of the alimentary tract.

In young embryos the tubules lie in the lateral part of the fat-body, elongating in a posterior direction, but later they turn anteriorly and ramify over the wall of the mesenteron (fig. 15, *Mt.*, Pl. 6).

#### RESPIRATORY SYSTEM.

This arises shortly after the development of the appendages in the usual manner from paired invaginations of the ectoderm, immediately anterior and lateral to the appendages in the thorax, and close to the antero-lateral margins of the abdominal segments.

The number of tracheal invaginations seems to be subject to considerable variation, even in embryos belonging to the same family. In the majority there appear to be two thoracic and eight abdominal spiracles, the prothorax and the posterior abdominal segments having no tracheal invaginations. This condition obtains in several Coleoptera (Graber, 1888), in *Chrysomela* (Strindberg, 1913), and also in *Apis* (Nelson, 1915) and in *Carausius* (Lehmann, 1926). *Pieris* (Eastham, 1930*a*) has also ten pairs of embryonic spiracles, but differs from the above-mentioned species in that the thoracic spiracles are located on the pro- and meta-thoracic segments, the mesothoracic invagination being suppressed. Some workers have observed nine pairs of abdominal spiracles, a condition which is considered by Imms (1925) to be the typical one. Heymons (1897), in addition to finding meso- and meta-thoracic and nine abdominal stigmata in *Lepisma*, observed that there were evidences of a rudimentary pair on the tenth abdominal segment.

From the above remarks it is evident that Coleopterous embryos in general seem to possess spiracles on the meso- and metathorax and the first eight abdominal segments, and it is

of interest to note that, while this arrangement was observed in *Euryope*, in *Corynodes* there are also slight invaginations on the ninth and tenth abdominal segments. Wheeler (1889) has described vestigial stigmata on the prothorax and on the tenth and eleventh abdominal segments of *Doryphora*, and, according to Eastham (1930 *a*), Toyama (1902) has observed ninth and tenth abdominal spiracles in the embryo of *Bombyx*, but these closed at the end of the embryonic period.

It would therefore appear from an analysis of the condition in Chrysomelid embryos alone that originally each body segment had a pair of spiracular invaginations, all of which may be traced in embryos like *Doryphora*. In other embryos (e.g. *Corynodes*) those of the prothorax and the eleventh abdominal segment have been suppressed, while in *Donacia* a further reduction has taken place on the tenth abdominal segment. The majority (*Lina*, *Chrysomela*, *Euryope*) resemble other types of embryos in having only ten pairs of spiracles, those of the meso- and metathorax and first eight abdominal segments still persisting.

The tracheal invaginations of *Corynodes* are much better developed than are those of *Euryope*. Those in the meso- and metathorax and first eight abdominal segments are relatively wide inpushings, which grow dorsally into the developing fat-body, where each divides into two branches both lying parallel to the surface of the body (fig. 18, Pl. 7). The ventral branch (*vb.*) is directed towards the ventral nerve-cord, while the dorsal branch (*db.*) extends almost to the cardioblasts (*cd.*).

The spiracles on the ninth and tenth abdominal segments (fig. 7, *sp.*, Pl. 6) are merely shallow inpushings of the lateral ectodermal wall. They can, therefore, only be regarded as rudimentary structures, probably indicating the occurrence of better-developed spiracles in some ancestral form.

#### TENTORIUM.

Concurrently with the development of the spiracles on the trunk segments there arises a series of paired invaginations in the head. These inpushings are much better marked in *Corynodes* than in *Euryope*, so that their gradual

development into the endo-skeleton of this region was more easily traced.

When they first appear the segments of the head are still in a relatively simple condition, the complicated changes, which subsequently result in the enlargement of the anterior part of the head, taking place some time later.

At their inception these inpushings are almost evenly spaced, and there seems to be no doubt that they constitute a definite metameric series. Four pairs of cephalic invaginations were observed in *Corynodes*, and after careful examination these are attributed to the antennary, mandibular, maxillary, and labial segments.

In possessing four pairs of ingrowths *Corynodes* agrees with the accounts given for other insect embryos, in which, however, there is still some confusion in regard to the segments on which they arise. Eastham (1930 *a*) has found that the embryo of *Pieris* agrees with that of *Apis* (Nelson, 1915) in that its four pairs of cephalic invaginations are intersegmental in position, and he concludes that they are important evidences of metamerism in the head region.

My own observations on the later embryos of *Corynodes* indicate that there is no essential difference in the condition of the tentorium of this species and *Pieris*, but examination of the earlier stages shows that in *Corynodes* a slightly different interpretation of their segmental arrangement is necessary.

When they first arise they are simple tubular ingrowths, lying at the anterior margins of the antennary, mandibular, maxillary, and labial segments. No invaginations occurred behind the labial segment, as in *Pieris*. During their later development they penetrate well into the head cavity, forming a complex supporting structure. Simultaneously the head is undergoing a very complicated series of changes, involving the backward migration of the mouth and the diminution in the size of the post-oral intersegmental regions. This reduction in the post-oral region results in a slight forward movement of the invaginations, so that they eventually come to lie at the posterior margin of the segment in front of the one on which



they were formerly situated. Thus the inpushing which at first was observed at the anterior border of the maxillary segment is later found on the posterior part of the mandibular segment, where it forms the strong mandibular apodeme.

In both *Euryope* and *Corynodes* the first invagination (fig. 20, *ta.*, Pl. 7) was observed dorso-laterally in the antennary segment, thus differing from that of *Pieris* (Eastham, 1930 *a*) in which it was located between the antenna and the mandible. They pass inwards and backwards to form the anterior arms of the tentorium, meeting a forwardly directed process from the body of the tentorium at the side of the stomodaeum.

The second pair of invaginations (fig. 21, *tm.*, Pl. 7) arise on the anterior margin of the mandibular segment and seem to correspond with the premandibular invaginations of *Pieris*. Unlike the latter they do not terminate against the wall of the mandibular segment, but passing inwards and downwards on each side of the stomodaeum and ventro-lateral to the cerebral ganglia, eventually meet the same process as the antennary inpushing. These invaginations are better developed in their early stages than in the older embryos.

Arising at almost the same point as the second invaginations is a pair of structures (fig. 21, *ca.*, Pl. 7), consisting of a solid mass of cells which apparently give rise to the corpora allata. Their origin in this segment is somewhat surprising as most investigators (Janet, 1899; Nelson, 1915; Eastham, 1930 *a*) find that they develop close to the ingrowth which gives rise to the mandibular apodeme. There is, however, no evidence of invaginations for the corpora allata in that position in *Corynodes*, and it must be assumed that in this respect *Corynodes* is exceptional.

These structures pass downwards and backwards, and in later embryos they are located close to the para-oesophageal commissure in the intercalary segment.

The invaginations on the maxillary segment (figs. 10, Pl. 6, 26, and 27, *tx.*, Pl. 7) are strongly developed, and from the first each is rather wide and slightly bifurcate. Each passes backwards, and as the dorsal part of the head develops its two branches extend dorsally at the sides of the stomodaeum, while

posteriorly a connexion seems to be established with the corpotentorium. These are the most conspicuous invaginations in the head, and they form an attachment for the strong mandibular muscles. This pair of cephalic structures corresponds with the mandibular apodemes of *Pieris* (Eastham, 1930 *a*), but, unlike the latter, no gland is developed at the extremity of either of its branches.

The posterior pair of invaginations (figs. 11 and 12, *tl.*, Pl. 6) develop at the anterior border of the labial segment. They grow inwards towards each other (fig. 11, Pl. 6), and passing over the sub-oesophageal ganglion (*sgn.*), but ventral to the stomodaeum (fig. 12, *st.*, Pl. 6), form the body of the tentorium, which in later stages gives off a pair of anterior processes uniting with the anterior tentorial arms.

Behind the labium no further cephalic invaginations were observed, the next being those at the anterior margin of the mesothorax, and it was therefore concluded that, as in *Euryope*, the silk and hypostigmatic glands or their homologues, which are recorded in Lepidopterous embryos, are absent in these Coleoptera.

Therefore, although in *Corynodes* the cephalic invaginations are rather different in position to those described by Eastham in *Pieris*, there can be no doubt that they are homologous structures. The metametric arrangement of these inpushings seems fully to justify the value attached to them by Eastham (1930 *a*) as affording further criteria for the interpretation of the segmentation of the head.

#### NEUROGENESIS.

Little need be said regarding the development of the nervous system in *Corynodes*, as it agrees in almost every detail with the accounts given for *Pieris* (Eastham, 1930 *a*) and *Euryope* (Paterson, 1932).

The brain and the ventral nerve-cord originate from the ectoderm shortly after the processes of gastrulation have been completed. Certain ectodermal cells (figs. 23 and 25, *nb.*, Pl. 7) on each side of the mid-ventral line become greatly enlarged, and are easily distinguished on account of the fact

that they stain less deeply than the other cells. These are the neuroblasts which by repeated divisions give rise to smaller daughter cells, thus forming the ganglia of the brain and nerve-cord. As a result of these cellular changes the ectoderm protrudes on each side of the mid-ventral line as the well-marked neural ridges. At the same time one or two neuroblasts are differentiated mid-ventrally, and these give rise to the middle cord (figs. 23 and 25, *mc.*, Pl. 7) which lies at the base of the deep depression of the neural groove. Intra-segmentally the cells of the middle cord later give rise to the transverse commissures which run into the ganglionic fibres developed from the ganglion cells, and thus the two ganglia of a pair are connected.

During the formation of the neural ridges the ectodermal cells lying on their ventral surface become markedly flattened and form a very thin one-celled layer, which gradually thickens as it passes into the broader ectoderm at the sides of the developing ganglia. This ectodermal layer (figs. 16 and 29, *ect.*, Pl. 7) then separates from the neural ridges, and the ventral chain of ganglia (figs. 16 and 29, *gn.*, Pl. 7) becomes a distinct system.

Eastham (1930 *a*) finds that the origin of the thin neurilemma, which becomes evident on the separation of the ganglia and the ventral body-wall, is difficult to trace in *Pieris*, but he considers that it develops from the outermost products of the neuroblasts. In *Corynodes* it seems to develop from the few elongate cells derived from the neuroblasts of the middle cord. These become evident shortly after the establishment of the middle cord (figs. 23 and 25, *nl.*, Pl. 7), and spreading obliquely upwards and outwards eventually surround the ganglion.

The sub-oesophageal ganglion (fig. 15, *sgn.*, Pl. 6) developing in the gnathite segments represents a fusion of three ganglia, and in addition to it there are thirteen pairs of ganglia in the trunk. A similar condition obtained in *Euryope*, and, as in that species, the ganglia of the eighth, ninth, and tenth abdominal segments fuse, so that in the older embryos (fig. 15, Pl. 6) the number of abdominal ganglia is reduced to eight, a number which is characteristic of most Chrysomelid larvae.

The brain develops in the cephalic region of the germ-band from lateral neural ridges in a manner similar to the nerve-cord

in the trunk region, the three pairs of ganglia which fuse to form it occurring in the labral, antennary, and intercalary segments. As in *Pieris* (Eastham, 1930*a*) and *Euryope*, the two ganglia of each pair are at first widely separated dorsally, but, as the conformation of the head gradually changes and its lateral and antero-dorsal walls develop, the ganglia increase markedly in size and become approximated dorsally. A deep mid-dorsal groove (fig. 26, *dg.*, Pl. 7) appears in the hypodermis, and from its cells nerve-fibres are developed and serve to connect up the two protocerebral or labral lobes. The nerve-cells of the third or tritocerebral ganglia give rise to commissures which pass out from each ganglion and unite with those of the opposite side under the stomodaeum, forming the post-oesophageal commissure (fig. 27, *trc.*, Pl. 7). The origin and development of this commissure is considered by Imms (1925) and Eastham (1930*a*) to indicate that the tritocerebral segment is post-oral in position. The tritocerebral ganglia also give rise to the para-oesophageal connectives which unite the brain with the sub-oesophageal ganglion.

#### STOMATOGASTRIC NERVOUS SYSTEM.

This develops, as in most insect embryos, from the cells of the wall of the stomodaeum, and forms a very obvious system even at a relatively early stage. It consists of frontal and posterior oesophageal (stomatogastric) ganglia joined by a fairly strong recurrent nerve. The frontal ganglion (fig. 21, *fgn.*, Pl. 7) is the most conspicuous part and is the first to be separated off from the stomodaeal wall. The oesophageal ganglion (figs. 26 and 27, *og.*, Pl. 7) seems to be separated rather earlier than the recurrent nerve (fig. 10, *rn.*, Pl. 6), and there can be no doubt that the whole system is evaginated from the stomodaeal wall. In this it differs from *Pieris* (Eastham, 1930*a*), where the stomatogastric system arises from two centres, an anterior one, giving rise to the frontal ganglion, and a posterior one, from which the oesophageal ganglion develops. The recurrent (stomatogastric) nerve is produced by cells proliferated from the two ganglia, and is, therefore, not differentiated from the stomodaeum as it is in *Corynodes*.

## DEVELOPMENT OF THE MESODERM.

The formation of the mesoderm in the trunk segments is essentially similar to that described for *Euryope*, the only difference being that it is on the whole much better defined in the present species. After the gastrulation period the cells of the lower layer spread out laterally dorsal to the ectoderm, and become differentiated into two lateral masses connected by a single layer of cells. These lateral cell aggregations in *Euryope* were interpreted as the rudiments of the mesoderm, while the connecting layer was considered to be the endoderm rudiment, opinions which are still retained after careful examination of preparations of *Corynodes*.

Most investigators have observed a similar stage in the various species which have been studied, and the majority are in agreement that the lateral longitudinal masses are incipient mesoderm elements. It is not surprising, therefore, to find that as regards the development of the structures derived from the mesoderm there is in general some uniformity of interpretation.

In *Corynodes* the mesoderm of the trunk segments, shortly after its differentiation from the lower layer, itself becomes marked off into dorsal and ventral portions by the appearance of coelomic cavities (fig. 25, *cc.*, Pl. 7). These are readily seen in the thoracic and first eight abdominal segments, in the latter of which they appear as rounded cavities, while in the former they are more elongate and slit-like. The coelomic cavities are not nearly so well developed as are those of *Carausius* in which Wiesmann (1926) describes and figures extremely well-defined cavities, each of which is obviously divided into a number of diverticula, the walls of which give rise to the different mesodermal structures. In *Corynodes* where the cavities are smaller there is no obvious differentiation of diverticula, and the growth of the mesoderm is consequently rather more difficult to trace.

The dorsal or splanchnic mesoderm (fig. 23, *spms.*, Pl. 7) is not sharply defined laterally from the ventral or somatic portion, but the development of the latter begins earlier and proceeds more rapidly.

Part of the somatic mesoderm (fig. 23, *sms.*, Pl. 7) is differentiated early to give rise to some of the body muscles. In the thoracic segments some of the cells of this portion elongate and pass into the leg-buds (fig. 24, *sms.*, Pl. 7), forming the muscles of these appendages. In all the trunk segments this mesoderm also gives rise to the ventral longitudinal muscles (fig. 29, *vlm.*, Pl. 7) on each side of the nerve-cord, while it is also apparent that the ventro-lateral muscles (fig. 29, *vl.*, Pl. 7), which are arranged in curved tracts similar to those described by Eastham (1930 *a*) in *Pieris*, are also derived from the somatic mesoderm. In these respects the development of the mesoderm of *Corynodes* agrees with that of *Pieris*, in which Eastham terms this the subsomitic mesoderm.

A difference is, however, observed in the development of the fat-body (figs. 18 and 29, *fb.*, Pl. 7), which arises as an early differentiation of the somatic mesoderm; its cells stain faintly and soon become vacuolated. It develops rapidly, spreading between the ventral muscles and invading the epineural sinus. In this way the mesoderm projects markedly into the yolk in the lateral regions of the growing embryo (figs. 18 and 29, Pl. 7). In *Pieris* Eastham finds that the fat-body develops from the somitic or dorsal portion of the mesoderm, while Wiesmann (1926) has observed that in *Carausius* it is proliferated off from a lateral coelomic diverticulum.

The dorsal muscles (fig. 29, *dln.*, Pl. 7) are separated off early from the lateral part of the somite, and it is difficult to determine whether they arise from the somatic or splanchnic mesoderm. They develop concurrently with the ventral system, and seem to belong rather to the lateral somatic mesoderm than to the splanchnic layer. Eastham (1930 *a*), however, finds that in *Pieris* they arise from the somitic mesoderm, which corresponds with the splanchnic mesoderm of *Corynodes*.

During and after the formation of the fat-body, the splanchnic mesoderm (fig. 18, *spms.*, Pl. 7) is sharply defined as a mass of deeply staining cells which project dorsally towards the yolk, from which they are separated by the narrow endoderm layer. The condition is very similar to that of Wiesmann's fig. 57, and is here indicated in figs. 18 and 24.

The cells of this mesoderm give rise to the musculature of the mid-gut, as in *Euryope*, and the cardioblasts which form the heart when the embryo closes dorsally are also derived from splanchnic elements.

#### THE CEPHALIC MESODERM.

The development of the mesoderm of the head is not so easily traced as is that of the thoracic and abdominal regions, its development becoming somewhat obscure during the remarkable changes in the head region.

In *Corynodes* the external segmentation of the embryo is almost coincident with the appearance of the mesoblastic somites, so that the condition in the early embryonic stages is fairly easy to determine. As has been previously mentioned, the head consists of six segments, the criteria for which are the occurrence of segmental ganglia and of paired appendages. The brain and sub-oesophageal ganglion are each composed of a fusion of three pairs of ganglia, representing the six paired cephalic ganglia, while the head appendages are the antennae, the vestigial intercalary (premandibular) appendages, the mandibles, maxillae, and labium, the labral lobes possibly indicating a pair of appendages on the first head segment.

A further criterion which is relied on in the determination of insect segmentation is the development of the mesoblastic somites. Wiesmann (1926) has been able to trace somites in all the head segments of *Carausius*, where they develop in a manner similar to that of the trunk segments, and concludes from this and other evidences that there are seven head segments. In the majority of insect embryos, however, considerable difficulty is experienced in the determination of the somites in the anterior head metameres.

In *Corynodes*, as in most species, the somites of the three gnathite segments are readily recognized. Those of the maxillary and labial segments are well defined, and in each a coelomic cavity is distinguishable. The mandibular somites are not so well marked, no well-defined cavity being observed. It is, therefore, doubtful if in *Corynodes* mandibular coelomic cavities are developed.

The mesoderm in these three segments resembles that of thoracic segments and gives rise to the muscles of the appendages and to the segmental muscles.

Anteriorly to the mandibular segment there is no single anterior mesoderm mass as described by Eastham (1930*a*) in *Pieris*, but instead three segments are indicated by the presence of three separate pairs of mesoderm elements. In the intercalary segment the lower layer differentiates as in other post-oral segments, but the lateral mesoderm (fig. 28, *ims.*, Pl. 7) has no well-marked cavity which could definitely be determined as coelomic. The fate of the intercalary mesoderm is doubtful in *Corynodes*. It spreads ventrally over the wall of the stomodaeum, but it could not definitely be said to give rise to the sub-oesophageal body as it does in *Pieris* (Eastham, 1930*a*; Henson, 1932). A structure (fig. 12, *sub.*, Pl. 6) consisting of pale cells seems to develop in the intercalary segment, and in later stages is found below the stomodaeum in sections passing through the maxillary and labial segments. Its cells present a close resemblance to those of *Pieris*, but they could not with any certainty be traced to an origin from the intercalary mesoderm.

The antennary mesoderm shows no characteristic somite formation, and no spaces could be observed. It forms a fairly uniform layer (fig. 32, *ms.*, Pl. 7) which gives rise to the muscles passing into the cavity of the antenna. Part of it also extends dorsally over the stomatogastric nervous system, after passing dorso-laterally between the brain and stomodaeum, and develops into the anterior aorta. In its position it differs from *Euryope*, where the aorta originates in the intercalary segment. In *Corynodes* the antennary mesoderm which gives rise to the aorta is never so well defined as it seems to be in *Pieris*, as figured by Eastham (1930*a*). It is always thin-walled, but its development, nevertheless, agrees with the accounts given by Wiesmann (1926) and Eastham (1930*a*) of the growth of the aorta in *Carausius* and *Pieris* respectively.

There is also no somite formation of the mesoderm in the labral segment, where it takes the form of a continuous layer



(figs. 28 and 31, *lms.*, Pl. 7) which is at first single-celled laterally, but two cells thick over the labral lobes into which additional cells are later proliferated, to form the labral adductor muscles. From this pre-oral mesoderm elements are separated off over the epipharyngeal region (fig. 28, *em.*, Pl. 7), where they give rise to the dorsal muscles of the pharynx.

#### THE GONADS.

The remarks regarding the development of the reproductive organs need only be brief, as it is similar to that of *Euryope* and other Chrysomelidae (Wheeler, 1889; Lécaillon, 1898; Hirschler, 1924-7). In probably all Chrysomelid embryos there is an early differentiation of germinal protoplasm (fig. 6, *gp.*, Pl. 6) at the posterior pole of the egg, and this foreshadows and determines the position of the genital rudiment (fig. 5, *gr.*, Pl. 6) which is located at the posterior extremity immediately after the formation of the blastoderm. In later development the cells of the rudiment are gradually carried inwards and forwards and come to lie in a dorso-lateral position among the mesoderm elements. As the lateral walls of the embryo grow around the yolk to effect the dorsal closure of the embryo, the developing gonads are carried more dorsally until they come to lie near the rudiments of the heart. In *Corynodes* the gonads (fig. 29, *gon.*, Pl. 7) are rather more anteriorly placed than those of *Euryope*, in which they were observed in the fifth and sixth abdominal segments. In the present species, as in *Pieris* embryos (Eastham, 1930 *a*) and Chrysomelid larvae, the genital cells were observed in the fat-body of the fourth abdominal segment.

#### THE ENDODERM.

Recently Eastham (1930 *b*) has given an excellent review of the position and the chief opinions in regard to the formation of the germ-layers in insects, with which the question of the endoderm is closely connected. This subject still offers many difficulties, and the question as to whether a true endoderm exists or not is still a controversial point. Most authors who maintain that the mesenteron wall is derived from the ectoderm

attach particular significance to the yolk-cells, since by some of them these cells are considered to be primary endoderm cells.

The majority of recent workers (Nelson, 1915; Eastham, 1930; Henson, 1932) support the bipolar origin of the endoderm rudiments. Eastham and Henson have shown conclusively that in *Pieris* there are definite bipolar mesendoderm rudiments, as they have called them, and Nelson in his lucid account of the development of *Apis* also shows that the mesenteron has a bipolar origin.

Leuzinger and Wiesmann (1926), in their very thorough examination of the development of *Carausius*, describe a slight ectodermal protuberance from the blind end of the proctodaeum, but state definitely that 'genaue Beobachtung zeigt jedoch auch hier deutlich dass dieser Ektodermfortsatz des Proctodäums nicht weiterwächst und mit der Bildung des sekundären Mitteldarmepithels nichts zu tun hat. Auch hier wird das Mitteldarmepithel infolge Substitution der Dotterzellenlamelle durch Blutzellen gebildet.'

This work is therefore highly interesting, in view of the fact that it represents a recent departure from the general tendency towards the acceptance of the bipolarity of the mesenteron rudiments.

Much work has been done on the embryology of various Coleoptera, and in regard to the development of the mid-gut there are, as in other groups, two sharply divided schools of thought, not only in connexion with the subject as applied to the whole order, but also in relation to its interpretation in closely allied species such as the various Chrysomelidae which have been investigated. A few authors such as Lécaillon (1898) assert that the mesenteron is derived from ectoderm, while the majority (Wheeler, 1889; Strindberg, 1913; Hirschler, 1924-7) show that it is an endodermal derivative, bipolar in origin.

In his recent review of the subject Eastham (1930*b*) points out the importance of the observations of Philpitschenko (1912), who has described the occurrence of a continuous median longitudinal endoderm rudiment joining up the anterior and posterior rudiments. Strindberg (1913) and Hirschler (1924-7) in the Chrysomelidae, and Heider (1928) in *Hydrophilus*

have also observed a similar condition, indicating that some Pterygotes pass through stages similar to those of the Apterygota, as described by Philpitschenko. Eastham is of the opinion that the continuous endoderm was the original condition and that it still obtains in the Apterygota, but that in the still more specialized Pterygotes it has almost disappeared, traces of it occurring only occasionally in certain species. As proof of his theory he cites the recent work of Mansour (1927) on *Calandra* and his own observations (1927) on the embryology of *Pieris*, but it must here be indicated, however, that the median longitudinal vestiges observed by Mansour and Eastham take no part in the formation of the mid-gut. These cells pass into the yolk and apparently assist in yolk liquefaction.

The occurrence of this median endoderm in certain Pterygotes is also significant in that it more or less supports Rabl's theory (1889; vide Graber, 1890) that the median part of the invaginated gastral groove gives rise to endoderm, while the lateral regions develop into mesoderm. Most investigators are inclined to discredit this theory, but in the opinion of the present writer a certain amount of interest and importance attaches thereto.

It has already been shown (Paterson, 1931, 1932) that in *Euryope* a median longitudinal layer of endoderm occurs as a result of gastrulation, and that the mid-gut epithelium takes its origin from this layer. It was partly with a view to substantiating this interpretation of the development of the mesenteron in *Euryope* that *Corynodes* was selected for embryological investigation, and there seems to be no doubt that there is a similar development in both species.

This single-celled layer of endoderm in both *Euryope* and *Corynodes* is differentiated early in development immediately after gastrulation, from the lower layer, that is before the invagination of the stomodaeum and proctodaeum. It remains intact and does not disintegrate into cells passing into the yolk. In all stages it forms a well-defined, if narrow layer (figs. 16, 18, 22, 23, &c., *end.*, Pl. 7), which is readily distinguished from the other layers by the fact that its nuclei are oval and horizontally directed, whereas the mesoderm nuclei are rounded and rather less deeply staining, while those of the ectoderm are oval

but vertically directed. This endoderm layer lies on the dorsal side of the embryo between the yolk and the developing nerve-cord, and, during the dorso-lateral growth of the embryo round the yolk, it is carried dorsally with the other layers and, enclosing the yolk, forms the wall of the mid-gut.

It is significant to note that this layer corresponds to the Blutzellenlamelle in *Carausius* (Leuzinger and Wiesmann, 1926), in which it gives rise not only to blood-cells, as its name denotes, but also to some transverse muscles and to the epithelial wall of the mid-gut.

In both *Euryope* and *Corynodes* certain cells are proliferated off from the endoderm layer into the epineural sinus (fig. 24, *eps.*, Pl. 7) and form the blood-cells (fig. 24, *bc.*, Pl. 7). There is also some indication that a transverse band of muscles (fig. 23, *tem.*, Pl. 7) is developed from it lateral to the nerve ganglion, but it is difficult to ascertain if these are entirely endoderm elements or if they are derived partly from the cells comprising the somatic mesoderm. It seems highly probable that it is identical with part of the Blutzellenlamelle, observed by Leuzinger and Wiesmann (1926) in *Carausius*, and with part of the median mesoderm of *Pieris* described by Eastham (1930 a).

In late embryonic life the mesenteron is a large structure filled with yolk and, as is indicated in fig. 15, Pl. 6, it becomes distinctly marked off into two large chambers which inter-communicate dorsally where the wall is folded and has the appearance of a small third chamber. Even at this stage an histological difference may be observed in the walls of the two portions of the mesenteron, the anterior chamber having much larger cells than the posterior region, which in comparison appears to be much thinner walled.

In larval Chrysomelids (Paterson, 1930) the mesenteron is also divisible into two regions, and in late embryos of *Euryope*, although the small dorsal chamber was not observed, the large anterior and posterior regions were similar to those of *Corynodes*.

It is therefore obvious from these embryological studies that, not only is the mid-gut in *Euryope* and *Corynodes*

definitely endodermal in origin, but that this single-celled median layer may be homologized with a similarly placed layer in other species belonging to both Apterygota and Pterygota. The chief difference lies in the fact that in other embryos the mid-gut arises either entirely from anterior and posterior rudiments, in which case the median layer disappears (Eastham, 1930 *a* and *b*), or else as Philpitschenko (1912), Strindberg (1913), and Hirschler (1924-7) find, this median layer may play only a small part in the formation of the mid-gut.

So far, very few studies have revealed that these bipolar rudiments may be lacking, and that the wall of the mid-gut may be derived from the median longitudinal layer. It is in this respect that the development of *Euryope* and *Corynodes* has proved most interesting.

#### SUMMARY.

An account is given of the embryology of *Corynodes pusis* Marsh, a Chrysomelid beetle feeding on *Asclepias fruticosa*. The development of this species is remarkably similar to that of *Euryope terminalis*, an allied species previously investigated by the writer (1931, 1932).

In both species there is a very early differentiation of the germinal protoplasm at the posterior pole, and the genital rudiment makes its appearance in this position immediately the blastoderm is completed.

The lower layer of cells is derived by proliferation and invagination of cells in the mid-ventral line, and this process is considered to represent the gastrulation phase of other invertebrate embryos. The cells resulting from this invagination differentiate into lateral mesoderm and median endoderm.

The mesoderm gives rise to appendicular and segmental muscles, fat-body, cardioblasts, and the muscle-layer of the mesenteron.

The epithelial layer of the mid-gut is considered to develop from the median endoderm, which gives no indication in either of these species of a bipolar condition. In this respect the development of these two species differs from that of other recently investigated insects, but shows some resemblance to

the condition described by Leuzinger and Wiesmann (1926) in the Orthopteran, *Carausius morosus*.

The development of the ectoderm is essentially similar to that of other insect embryos. The stomodaeum and proctodaeum are invaginations of the ectoderm, as are also the respiratory and excretory systems. In addition to the two pairs of thoracic and eight pairs of abdominal spiracles observed in *Euryope*, there are vestigial spiracular invaginations on the ninth and tenth abdominal segments of *Corynodes*. In both species the Malpighian tubules arise as three separate pairs of outgrowths of the wall of the proctodaeum, and there is no indication that any part of their wall is derived from the endoderm, an opinion recently expressed by Henson (1932) when describing the condition in the embryo of *Pieris*.

The tentorium is rather better developed in *Corynodes* than in *Euryope*, and in the embryo four pairs of cephalic ectodermal invaginations were observed to arise in a series one behind the other on the antennary, mandibular, maxillary, and labial segments.

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## EXPLANATION OF PLATES 6 AND 7.

## LETTERING OF FIGURES.

*a.*, aorta; *af.*, amniotic fold; *am.*, amnion; *an.*, anus; *ap.*, appendage; *at.*, antenna; *bc.*, blood-cell; *ca.*, corpora allata; *cb.*, cerebral ganglion; *cc.*, coelomic cavity; *cd.*, cardioblasts; *cp.*, crop; *cr.*, chromosomes; *db.*, dorsal branch of trachea; *dg.*, dorsal cephalic groove; *dln.*, dorsal muscles; *ect.*, ectoderm; *em.*, epipharyngeal mesoderm; *end.*, endoderm; *eps.*, epineural sinus; *er.*, embryonic rudiment; *ex.*, extra-embryonic blastoderm; *fb.*, fat-body; *fg.*, frontal ganglion; *gg.*, gastral groove; *gn.*, ganglion; *gon.*, gonad; *gp.*, germinal protoplasm; *gr.*, genital rudiment; *il.*, ileum; *ims.*, intercalary mesoderm; *ll.*, lower layer; *lp.*, labial palp; *lpt.*, lateral plate; *lr.*, labrum; *lms.*, labral mesoderm; *m.*, mouth; *mc.*, middle cord; *md.*, mandible; *mes.*, mesenteron; *mp.*, middle plate of cells; *ms.*, mesoderm; *Mt.*, Malpighian tubule; *mx.*, maxilla; *mzp.*, maxillary palp; *nb.*, neuroblast; *nl.*, neurilemma; *oes.*, oesophagus; *og.*, oesophageal ganglion; *pb.*, polar body; *pc.*, protocerebral lobe of brain; *per.*, periplasm; *ph.*, pharynx; *pn.*, pronucleus; *pp.*, polar plasm; *proc.*, proctodaeum; *ps.*<sub>1</sub> and *ps.*<sub>2</sub>, first and second polar spindles; *rc.*, rectum; *rn.*, recurrent nerve; *s.*, spindle; *ser.*, serosa; *sgn.*, sub-oesophageal ganglion; *sms.*, somatic mesoderm; *sp.*, spiracle; *spms.*, splanchnic mesoderm; *st.*, stomodaeum; *sub.*, sub-oesophageal body; *ta.*, first cephalic or antennary invagination; *tc.*, tritocerebrum; *tem.*, transverse muscle from endoderm; *tl.*, fourth cephalic or labial invagination; *tm.*, second cephalic or mandibular invagination; *trc.*, post-cephalic commissure; *tx.*, third cephalic or maxillary invagination; *v.*, oesophageal valve; *vb.*, ventral branch of trachea; *vl.*, ventro-lateral muscle; *vlm.*, ventral longitudinal muscle; *vm.*, vitelline membrane; *vt.*, vitellophag; *yk.*, yolk.

## PLATE 6.

Fig. 1.—Longitudinal section of equatorial region at oviposition, showing first polar spindle. ×985.

Fig. 2.—Longitudinal section of equatorial region 30 minutes after oviposition. ×985.



Fig. 3.—Longitudinal section 2 hours after oviposition, showing first polar body and female pronucleus.  $\times 350$ .

Fig. 4.—Longitudinal section of middle region 10 hours after oviposition.  $\times 985$ .

Fig. 5.—Longitudinal section of posterior pole 30 hours after oviposition, by which time genital rudiment is established.  $\times 610$ .

Fig. 6.—Longitudinal section of posterior pole at oviposition, passing through the germinal protoplasm.  $\times 610$ .

Fig. 7.—Transverse section through ninth abdominal spiracle in same embryo as Fig. 18.  $\times 350$ .

Fig. 8.—Transverse section at 72 hours, showing the differentiation of the embryonic rudiment from the extra-embryonic blastoderm.  $\times 130$ .

Fig. 9.—Transverse section of 72-hours-old egg, in which the middle plate is becoming separated from the lateral plates.  $\times 160$ .

Fig. 10.—Transverse section of head of 9-day-old embryo showing third cephalic invagination.  $\times 350$ .

Figs. 11 and 12.—Transverse sections through the posterior cephalic invagination on the ninth and thirteenth day respectively.  $\times 350$ .

Fig. 13.—Transverse section through posterior end of 4-day-old egg, showing dorsal curvature of embryo.  $\times 160$ .

Fig. 14 A and B.—Sections showing first origin of Malpighian tubules from ectodermal proctodaeum.

Fig. 15.—Longitudinal section of embryo prior to hatching, constructed from several sections, and showing the parts of the alimentary canal.  $\times 120$ .

#### PLATE 7.

Fig. 16 A-E.—Consecutive transverse sections through the eighth abdominal segment of a 9-day-old embryo to show the development and arrangement of the Malpighian tubules.  $\times 350$ .

Fig. 17.—Transverse sections of yolk-nuclei, indicating their arrangement in groups.  $\times 550$ .

Fig. 18.—Transverse section through anterior abdominal spiracle to show the division of the trachea into two branches.  $\times 350$ .

Fig. 19.—Portion of Fig. 9.  $\times 550$ .

Fig. 20.—Transverse section of head of 8-day-old embryo passing through first cephalic invagination and roof of stomodaeum.  $\times 350$ .

Fig. 21.—Transverse section of 9-day-old embryo showing the second cephalic invagination.  $\times 350$ .

Fig. 22.—Transverse section through posterior end of 6-day-old egg in which the three embryonic layers—ectoderm, endoderm, and mesoderm—are differentiated.  $\times 350$ .

Fig. 23.—Transverse section through third thoracic segment to show the development of the mesoderm and the appearance of the neurilemma.  $\times 370$ .

Fig. 24.—Transverse section of maxillary segment in an embryo 7 days

old; the epineural sinus has made its appearance and the somatic mesoderm is seen passing into the appendage.  $\times 370$ .

Fig. 25.—Transverse section of abdominal segment of 5-day-old embryo showing the early differentiation of the mesoderm.  $\times 370$ .

Figs. 26 and 27.—Transverse sections through the third cephalic invagination in a 13-day-old embryo.  $\times 350$ .

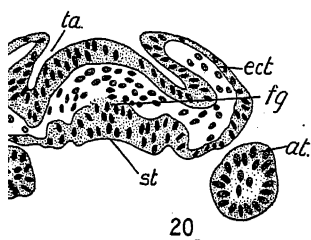
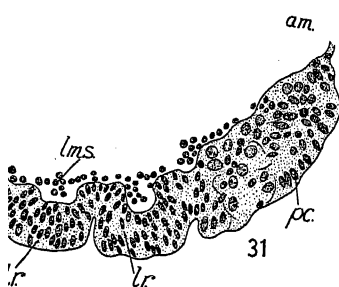
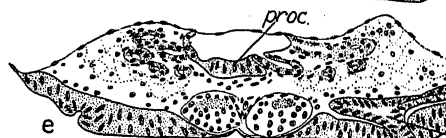
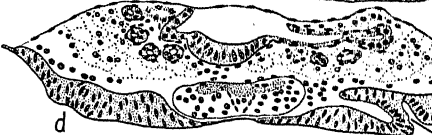
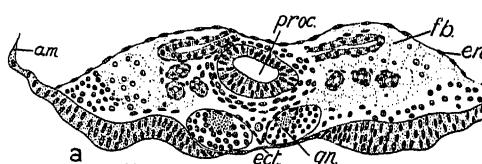
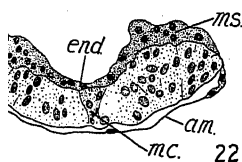
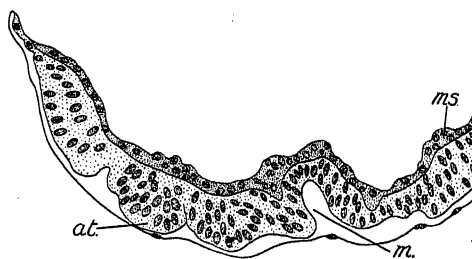
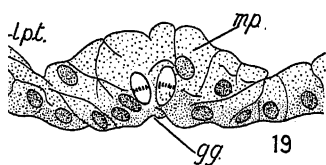
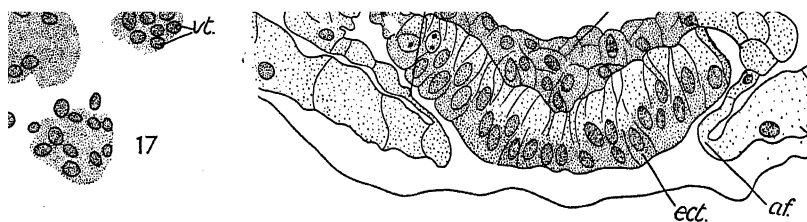
Fig. 28.—Longitudinal section through the head of an 8-day-old embryo, to show the condition of the mesoderm in the oral region.  $\times 350$ .

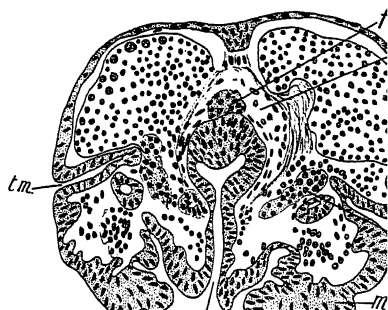
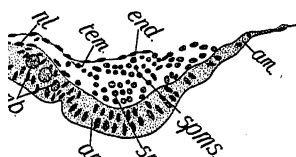
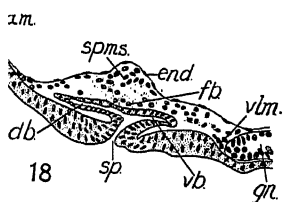
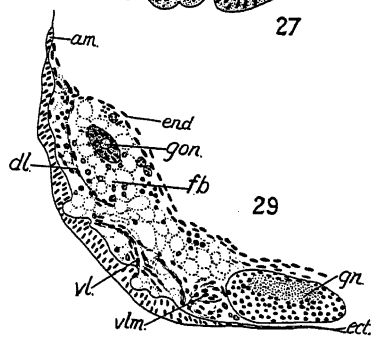
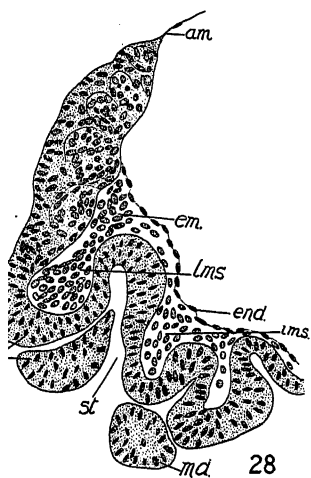
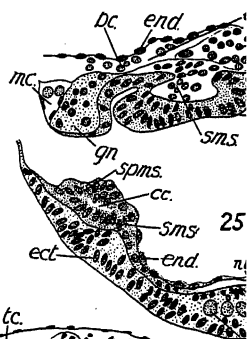
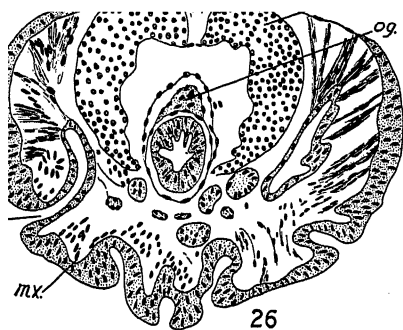
Fig. 29.—Transverse section through the fourth abdominal segment in an embryo 13 days old, passing through the gonad and indicating the arrangement of the segmental muscles.  $\times 350$ .

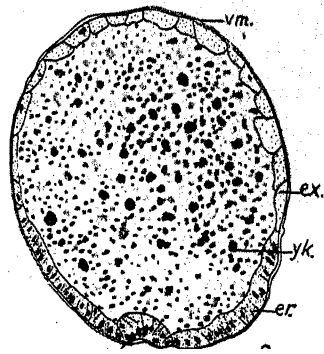
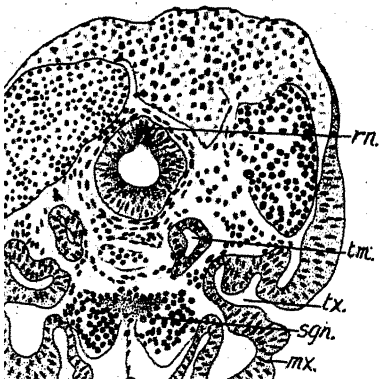
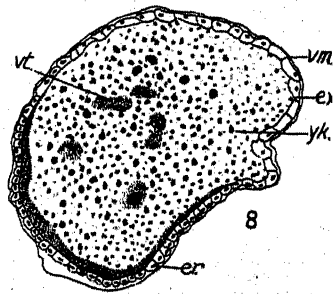
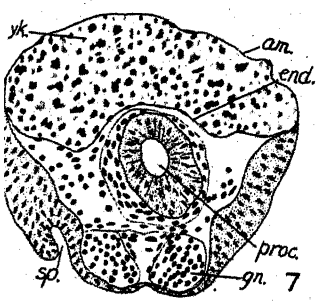
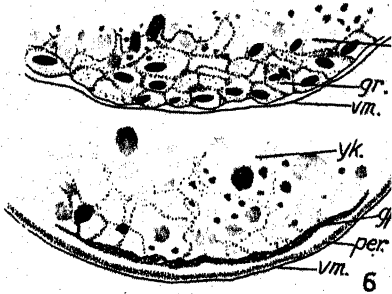
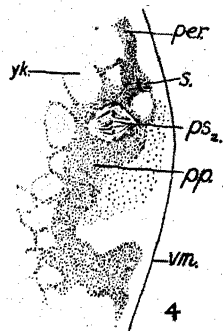
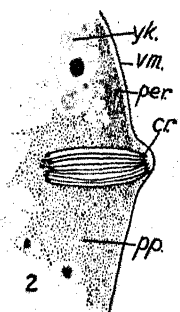
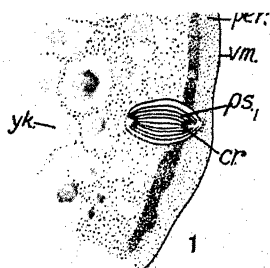
Fig. 30.—Transverse section through middle region of 4-day-old egg, showing the development of the amniotic folds.  $\times 550$ .

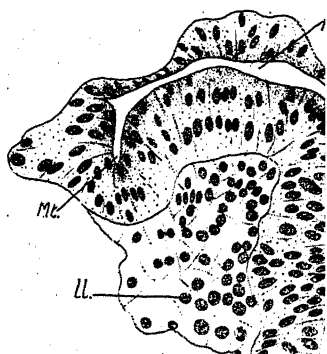
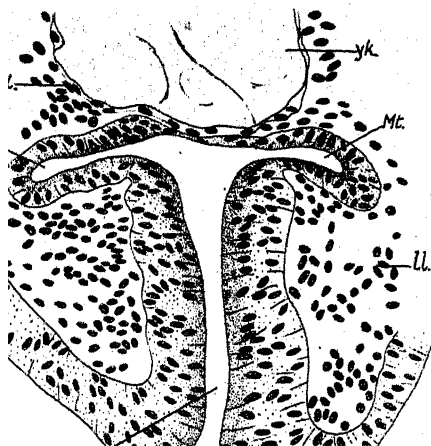
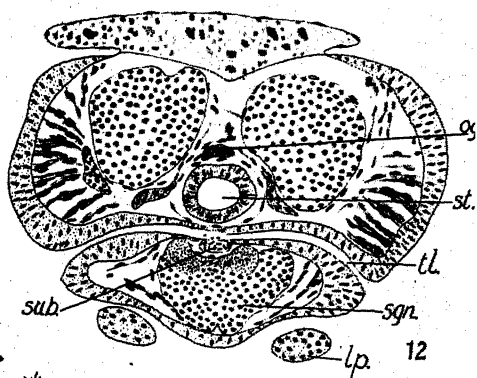
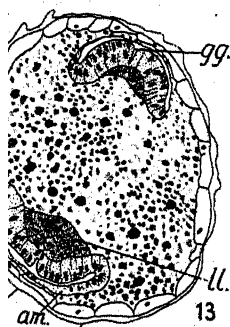
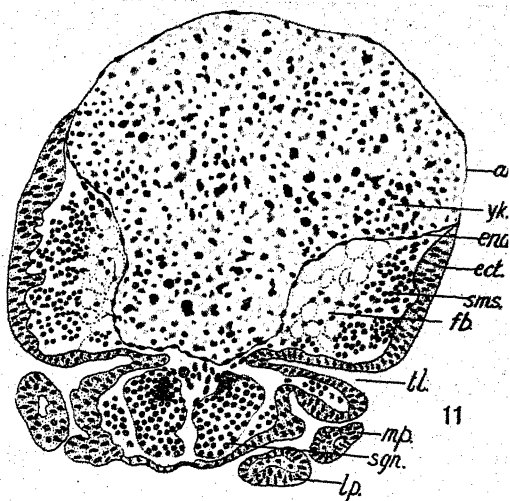
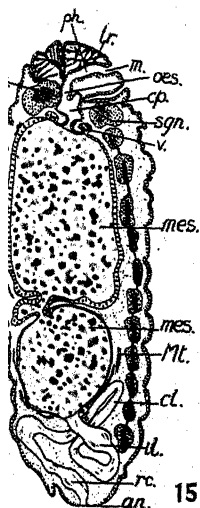
Fig. 31.—Transverse section passing through labral segment and showing that the labrum is originally bilobed.  $\times 370$ .

Fig. 32.—Transverse section of anterior end of embryo shortly after the invagination of the stomodaeum.  $\times 350$ .











# The Formation of the Hen's Egg.<sup>1</sup>

## PARTS I-IV.

By

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Jassy, Roumania.)

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With 20 Text-figures.

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I. MORPHOLOGY AND METABOLISM OF  
YOLK-FORMATION.

## INTRODUCTION.

YOLK-FORMATION is an extremely complex process.

The yolk seen as the yellow part of the egg is the result of a great number of chemical and morphological changes. The latter have been very well described in many works on the behaviour of intra-ovular organelles (mitochondria and Golgi elements) during yolk-formation. The former have been the subject of only very few works up to the present.

Histochemically, yolk-formation has been much better studied. But the majority of these studies has been limited to the examination of fat, non-saturated acids, lipoids, glycogen, and, in more recent times, of thymonucleic acid. The behaviour of saturated fatty acids, cholesterol, proteins, and salts has not formed the subject of any histochemical study.

We propose to fill these gaps. As material for study we have chosen the hen's egg.

Up to now we have studied histochemically the behaviour of the cholesterol, iron, fixed ash, nuclear (thymonucleic acid), and plasmal reactions, at the time of yolk-formation of the hen's egg. In the present work we shall show results obtained from the study of proteins during this process.

Most of the methods used by us during this work have been applied for the first time to the study of yolk-formation. Knowing the defects of the majority of histochemical methods, we have chosen those which most resemble reactions used in chemistry.

For the purpose of introducing this study into the collection of researches made on yolk-formation, the study of proteins is preceded by two other chapters: one relating to the morphology

and metabolism of the hen's egg during yolk-formation, another relating to the morphological and chemical changes which the follicular epithelium undergoes in the course of yolk-formation.

The aim of our work is to produce a contribution to the histophysiology of yolk-formation. We shall consider histochemistry as the connecting-link between histology and chemistry. Thanks to the chemical reactions adopted, histochemistry allows of deep penetration into intracellular metabolism, which is the basis of all morphological phenomena.

### THE GROWTH OF THE OVULE.

The growth of the hen's ovule has occupied the attention of embryologists for a long time. They have tried to divide this growth into various periods, making use of either a morphological, histophysiological, or chemical standard. Each one of these divisions considered only one aspect of the process of the ovule's growth, neglecting the others. There still remains, however, a definite uncertainty as to the sizes of the hen's egg during the different stages of yolk-formation.

In this chapter we shall attempt to co-ordinate and systematize the various classifications of yolk-formation, give a general rapid view of all aspects shown by this process, and establish the relations which exist between the size of the ovules and the phases of yolk-formation.

We shall briefly describe the maturation of the hen's egg, in order to be able to stress later, at greater length, the changes to be seen in the ovule during its development, as well as the speed with which it grows during this time.

### Development of the Oocyte.

Towards the tenth day of incubation, the cords of Pflüger are seen to appear in the ovary of the hen's embryo. From these cords the primary oogonic cells, which have great powers of multiplication, are isolated. This multiplication does not follow the same pattern in the whole ovary: in the middle of the organ it stops towards the fifteenth day of incubation; at the periphery of the organ the multiplication of oogonia goes on to the end of incubation (d'Hollander).

The primary oogonia give birth to secondary oogonia. These cease multiplying and become first-order oocytes.

The first-order oocyte undergoes a lengthy development. It passes through a series of great nuclear and cytoplasmic changes. These changes begin as early as the differentiation of the oocyte during incubation, and continue up to adult age of the hen.

During this time the oocyte increases in size considerably and reaches from 15 to 20 microns in diameter (fifteenth day of incubation) to 3-3.5 cm. diameter (end of growth), increasing, therefore, to approximately two thousand times its original diameter.

Having reached the limit of its growth, the ovule is expelled into the oviduct (dehiscence of the follicle). Shortly before this dehiscence, the phenomena of maturity have occurred in the nucleus of the oocyte, and the primary oocyte is changed into the secondary oocyte. In the oviduct the oocyte is fertilized; later it is surrounded by other layers and membranes which characterize the laid egg.

#### Extra- and Intra-follicular Periods.

Shortly after formation, the primary oocyte is surrounded by a continuous layer of flat cells. The whole constitutes an ovarian follicle.

It therefore comes about that the evolution of the oocyte is divided into two phases: first, the extra-follicular period, and second, a period during which the oocyte is isolated from the rest of the ovary by the follicular cells which completely surround it (intra-follicular period).

The Extra-follicular Period is very short. It characterizes the embryonic life of the oocyte. This period begins the fifteenth day of incubation of the hen's egg, and finishes 4 to 6 days after the hatching of the young chicken.

During the extra-follicular period, important changes take place near the oocyte's nucleus.

The Intra-follicular Period begins several days after hatching, and ends at the dehiscence of the mature ovule.

Nuclear changes continue for a certain length of time at the beginning of the intra-follicular period, but later are very diminished up to the end of this period, when nuclear maturation takes place. During this period the ooplasm increases in size considerably; moreover, it undergoes a series of changes which make possible the accumulation of yolk. The oocyte is changed from a hardly distinguishable cell to a cell remarkably noticeable both morphologically and chemically.

The duration of the extra-follicular period is from 10 to 12 days. The intra-follicular period lasts from several months to several years, it having been ascertained that certain ovules reach maturity more quickly, while others take longer to reach this stage.

### Nuclear Changes.

The nuclear changes in the oocyte during the extra-follicular period have been extremely well described by d'Hollander in a work which has become classical.

*Oogonia*.—At the level of these cells d'Hollander has not found any nuclear changes.

*Oocytes*.—These changes are not seen until after the change of the secondary oogonium into the first-order oocyte. These changes are summed up in the following table:

TABLE 1.

Nuclear changes of the primary oocyte during the extra-follicular stage, and at the beginning of the intra-follicular stage (according to d'Hollander's data).

<i>Days of Incubation.</i>	<i>Nuclear Type.</i>	<i>Name of Stage.</i>	<i>Appearance of Nucleus.</i>
A. Extra-follicular Period.			
10-15	b	Resting	Reticular.
15-19	c	..	Central chromatin.
16-19	d	Leptotene	Small cluster; nucleolus.
17-19	e	Zygotene	Synapsis.
18-20	f	"	Synapsis extended.
19-21	g	Pachytene	Thick thread.
21-21+4	h	..	Peripheral thread.
21-21+4	i	Diplotene	Longitudinal split; peripheral nucleolus.
21-21+3	j	..	Reticulum.

## B. Intra-follicular Period.

<i>Days after Hatching.</i>	<i>Nuclear Type.</i>	<i>Name of Stage.</i>	<i>Appearance of Nucleus.</i>
4	j	Stage 1	Reticulum; nucleoli.
6	j	„ 2	Thickening of chromatic rings.
6-20	j	„ 3	Varicous rings; nucleoli; nuclear membrane; double contour.
6-20	j	„ 4	Barbed rings (plumose); voluminous nucleoli.

d'Hollander's study stops at Stage 4 of type 'j' nuclei. Sonnenbrodt, Marie Loyez, Harper, and Modeste van Durme have studied later stages. Certain oocytes remain at Stage 'j' for years. Other ovules destined for approaching maturity undergo the cycle of change proper to the period of the ovule's growth.

**Nucleoplasmatic Ratio.**—During Stage 4, type 'j', the ovule's nucleus forms two-thirds of the whole cell (ovules from 30 to 50 microns diameter); later (ovules from 50 to 70 microns diameter) the nucleus constitutes only half of the cell. The nucleoplasmatic ratio decreases according to the development of the oocyte (Brambell).

In the following stages the ovular nucleus continues to increase in size, but this increase is, in proportion, much less than in the ooplasm. The nucleoplasmatic ratio diminishes continually during the growth of the hen's egg.

During the extra-follicular stage the nucleus is situated at the centre of the oocyte. Shortly after the beginning of the intra-follicular stage, the nucleus moves to the periphery of the ovule. At the end of the ovule's growth the nucleus is found only a few microns from the vitelline membrane, having therefore only a very superficial position.

## Nuclear Changes in the Intra-follicular Period.

As a general rule, after hatching, the nuclear structure is more and more simplified, so that towards the end of the ovule's growth it is only represented by a few very shortened barbed segments.

Another equally important factor is the chromatin's change in colouring. This, strongly basophil at the beginning, becomes less so during the ovule's development. We shall return to this question when we come to nuclear reaction.

Maturation occurs at the end of the ovule's growth. The chromosomes of the hen's egg are small and shortened (van Durme).

To sum up: nuclear changes are more intense during the extra-follicular period and at the end of the intra-follicular period. During all the rest of the intra-follicular period nuclear changes are very slight.

TABLE 2.

Changes observed in the nucleus of the hen's egg during the period of intra-follicular growth.

<i>Diameter of Ovules (in mm.).</i>	<i>Chromatin.</i>	<i>Appearance of Nucleus.</i>	<i>Authors.</i>
0.1-0.2	Large varicous rings, transversely streaked	Large, retracted	van Durme; Brambell.
0.3-0.9	Part disaggregation of barbed segments	Increase in nuclear volume, nuclear juice, and number of karyosomes	Ditto.
1.0-2.0	Part disaggregation of barbed segments	..	van Durme.
2.0-4.0	Reduction in length of barbed segments	Double contour membrane; rare nucleolus; nuclear volume increased	Ditto.
4.0-6.0	Barbed segments persist <sup>1</sup>	Disappearance of nucleolus	Ditto.
6.0-10.0	Number of barbed segments very diminished	Nucleus plano-convex applied against vitelline membrane	Ditto.
10.0-30.0	Rings or lozenges concentrated at centre of nucleus	Ditto	Ditto.
30.0	Compact rods or chromatin balls	Ditto	Ditto.
30.0-35.0	First nucleus spindle of maturation	Disappearance of nuclear membrane	van Durme; Harper. <sup>2</sup>
Dehiscence <sup>3</sup>	Second nucleus spindle of maturation	..	Ditto.

<sup>1</sup> The barbed segments disappear, according to Loyez and Sonnenbrodt, an opinion disputed by van Durme.

<sup>2</sup> Harper has studied the mature pigeon's egg.

<sup>3</sup> M. van Durme has studied nuclear phenomena in added hens' eggs. We have given his results to dimensions of ovules of the Orpington and Red Island types, used mostly by us.

## MORPHOLOGY OF YOLK-FORMATION.

## Phases of Yolk-formation.

During the period of the ovule's development the ooplasm undergoes great morphological and chemical changes. Its appearance changes several times during yolk-formation of the hen's egg. When describing these changes several authors have tried to establish a division into various periods of yolk-formation. But the majority of these divisions are more or less arbitrary. For the hen's egg, the division which most conforms to the known facts of histophysiology is that formulated by van Durme in 1914.

According to this author yolk-formation presents three phases. The first is characterized by the appearance of fat spheres in the ooplasm; the second by the appearance of transparent vacuoles and by the formation of yolk inside the vacuoles; the third by the appearance of yellow yolk-globules, the formation of latebra, and the nucleus of Pander.

## First Phase of Yolk-formation.

At the beginning of this phase a formation, which is known as 'the vitelline body of Balbiani', makes its appearance in the ooplasm. This formation, which seems to be composed of several layers,<sup>1</sup> appears fairly soon in the evolution of the ovule, but it is more easily seen in ovules from 70 to 150 microns diameter (Text-fig. 1).

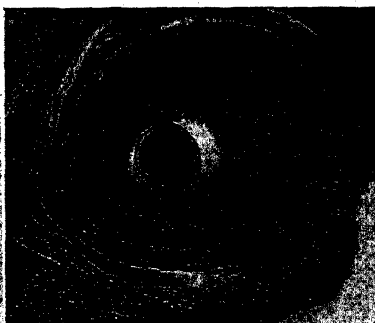
In larger ovules the body of Balbiani becomes subdivided, and ends by disaggregating. It is no longer found in ovules of 300 microns diameter (van Durme). In ovules from 300 to 1,000 microns diameter a layer of fat globules is found at a little distance from the periphery of the ovule (Text-fig. 2). The remainder of the ooplasm assumes a finely granulated appearance under the influence of fixatives used by us (formol, alcohol, acetic sublimate, &c.).

The diameter of the fat globules decreases as the ovule

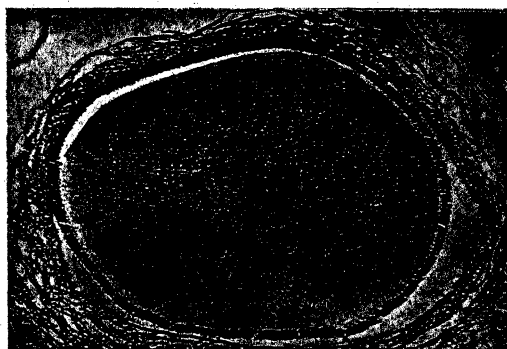
<sup>1</sup> In a later work we propose to return in greater detail to the subject, as well as to the number of layers which compose the Balbiani body.



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

## TEXT-FIGS. 1-3. First Phase of Yolk-formation.

Fig. 1.—Ovary No. 24. Fixative—alcohol; basic proteins after HCL 5 per cent. Bordeaux B. Ovule of  $70 \times 120$  microns diameter ( $6 \times D$ ). Ovoplasm colourless. Balbiani body (BB.), chromatin (ovule nucleus) and follicular epithelium clearly visible.

Fig. 2.—Ovary No. 81. Fixative—formalin 10 per cent.; stain Nile blue. Ovule of  $414 \times 522$  microns diameter ( $6 \times A$ ). Granulo-fatty cortical layer clearly seen (CGG.).

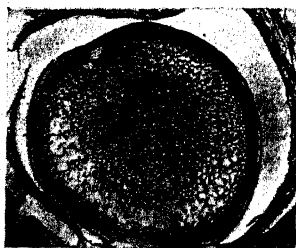
Fig. 3.—Ovary No. 24. Fixative—alcohol 96; acid proteins (Unna's method). Ovule of  $540 \times 810$  microns diameter ( $6 \times A$ ). Follicular epithelium more intensely coloured than other elements of ovule and its membranes. The granulo-fatty cortical layer is, at this stage, in a state of disaggregation.



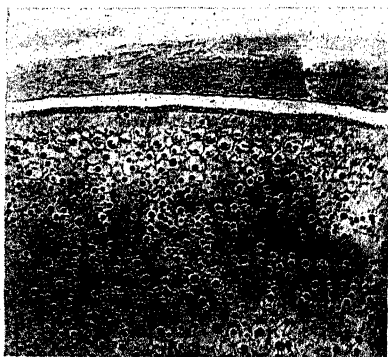
develops. This layer disappears so as to be only dimly visible in ovules of 1-2 mm. diameter (Konopacka, ourselves) (Text-fig. 3).

### Second Phase of Yolk-formation.

Transparent vacuoles begin to appear in the ooplasm. These become more and more numerous, and in ovules of 2 mm.



TEXT-FIG. 4.



TEXT-FIG. 5.

### TEXT-FIGS. 4-5. Second Phase of Yolk-formation.

Fig. 4.—Ovary No. 42. Fixative—alcohol; basic proteins after HCL 5 per cent. (Bordeaux B) (oc. comp.  $2\times A$ ). Ovule of  $2.5\times 2.8$  mm. diameter. Germinal vesicle seen in an eccentric position, situated between granular cortical layer (GCL.) and central vacuolar layer (VR.). Latter layer occupies entire centre of ovule.

Fig. 5.—Ovary No. 78. Fixative—alcoholic digitonine, inclusion in gelatine (Leulier and Noel's method for histochemical detection of cholesterol); microphotography with Nicols parallels; section not coloured. Ovule of  $4\times 5.5$  mm. diameter ( $6\times A$ ). On periphery of ovule, granular cortical layer is seen, under which a thin layer of white yolk and a very thick layer of primordial yolk occurs in a clearly visible vacuolar web.

diameter they constitute a layer which takes up the whole centre of the ovule. At the periphery of the ovule there remains only a narrow layer which keeps the granular appearance of the ooplasm in the preceding stage (granular cortical layer) (GCL. Text-fig. 4).

The vacuoles seen at the time of the second phase of yolk-

formation are not of the constitution of fat. They take neutral red well (Ram Saran Das), but are coloured neither by Sudan III nor by Nile blue, nor any other fat stains (Parat).

Van Durme divides the second phase of yolk-formation into two stages. Stage A, characterized by the presence of the central vacuolar layer; during the second stage, Stage B, round yolk-globules are precipitated into the transparent vacuoles (Text-fig. 5). The diameter of these yolk-globules is larger in the cortical region of the vacuolar layer (van Durme's exoplasmic layer); in the central region of the ovule globules of smaller diameter are found (van Durme's endoplasmic layer).

We have called the yolk which is formed during the second phase of yolk-formation primordial yolk, to distinguish it from the forms of yolk which will make their appearance later.

### Third Phase of Yolk-formation.

The third phase of yolk-formation is characterized by the appearance of yellow yolk and white yolk.

The arrangement of the different layers of yolk is not the same as during the second phase of yolk-formation. At the centre of the ovule a round vitelline formation from 4 to 6 mm. diameter is to be found, called Purkinje's latebra (Text-fig. 10). The latebra is connected with the ovule's surface by a cone of yolk in little globules, the nucleus of Pander.

The germinal vesicle (nucleus ovule) rests on the nucleus of Pander. It is, at this stage, peripheral. The remainder of the ovule consists of yellow yolk, except for a peripheral region where white cortical yolk is to be found.

That is the appearance of the hen's egg at the end of its growth. But we have seen that between this appearance and that of ovules during the second phase of yolk-formation there is no sudden transition. There is an intermediate phase which has sufficient distinguishable characteristics to deserve separate description.

To preserve the nomenclature of van Durme we have called it Stage A of the third phase. The ovule with white cortical yolk, yellow yolk, latebra, and nucleus of Pander constitutes Stage B of the third phase.

TEXT-FIG. 6.



TEXT-FIG. 8.



TEXT-FIG. 7.

TEXT-FIG. 9.



TEXT-FIGS. 6-9. Third Phase of Yolk-formation.

Fig. 6.—Ovary No. 77. Fixative—alcohol 96; basic proteins coloured by Bordeaux B—alum haematin (Unna's method). Ovule of 4 mm. diameter ( $6\times D$ ). Radiated zone under follicular

Stage A of the Third Phase.—In ovules about to enter upon the third phase of yolk-formation we have noted the appearance of a variety of globules at the periphery, not round in shape, and homogeneous, like primordial yolk-globules. The new globules consist of a fundamental substance, to use Dubuisson's expression, and a small number of spherular inclusions (Text-fig. 7). Under the action of fixatives these inclusions collect at one of the poles of the globule, giving this a semi-lunar appearance.

The globules with inclusions are the white yolk-globules, such as Balfour describes and indicates in his treatise on embryology.

The majority of earlier authors (His, Miescher, Balfour) and some modern authors (Dubuisson, Loyez, Konopacki, and Konopacka, Mary Guthrie, Brambell, &c.) make no distinction between white yolk-globules and primordial yolk-globules.

epithelium (in pseudo-stratified phase). Granules of various sizes are seen in granular cortical layer, very thin in this ovule. In sub-jacent region are globules of intravacuolar primordial yolk (second phase of yolk-formation).

Fig. 7.—Ovary No. 36. Fixative—alcohol 96; reaction of potassium (Macallum's method). Ovule of  $6 \times 7$  mm. diameter ( $7 \times D$ ). Appearance of granular cortical layer similar to that in preceding figure. Below it are several globules of white yolk (wy.), with spherular intraglobular inclusions. Under action of fixative, these inclusions are gathered together at one pole of globule. Globules of white yolk are formed of material diffused into ovule without going through primordial yolk stage. Below white yolk-globules are globules of primordial yolk (py.).

Fig. 8.—Ovary No. 77. Fixative—alcohol 96; basic proteins coloured by Bordeaux B—Unna's alum haematin (Unna's method). Ovule of  $6 \times 8$  mm. diameter ( $15 \times A$ ). Under granular cortical layer is a thin layer where globules of primordial yolk are mixed with globules of white yolk; below this zone is a thick layer of white yolk and a layer of primordial yolk. Owing to fixative, inclusions of the white yolk-globules are gathered up at one pole of globule, giving it a semilunar appearance. In membranes of this ovule juxta-epithelial layer (JEL.) is more intensely coloured than the internal theca (IT.).

Fig. 9.—Ovary No. 73. Fixative—alcohol; basic proteins after action HCL 15 per cent. Coloration Bordeaux B (Unna's method). Ovule of 20 mm. diameter ( $6 \times A$ ). Every element took Bordeaux B intensely. Under follicular epithelium, globules of smaller size are seen (white cortical yolk-globules). Among yellow yolk-globules, smaller-sized globules also seen, but no stratification. Removal of paraffin from section has raised many yolk-globules.

Riddle considers the yolk of the second phase of yolk-formation (our primordial yolk) as pure white yolk. Marie Loyez has shown these globules in layer C of Figs. 138 and 140. Dubuisson has seen and described them as an intermediate stage between the round globules of yolk (our primordial yolk) and yellow yolk. Brambell shows the white yolk-globules in the text of his article, and designates the intraglobular inclusions as 'mitochondrial-yolk' (M-Y), and the rest of the globule as 'mitochondrial-ground-cytoplasmic-yolk' (M-C-Y). Guthrie has also observed them in the egg of *Fundulus*.

According to Konopacka, the vacuole where the globule of primordial yolk is precipitated constitutes the outline of the vitelline globule. The primordial yolk-globule is described as 'violet globule' because of its affinity for haemalum. The inclusions of the white yolk-globules are referred to by Konopacka as 'phospho-protein globules', being a mixture of proteins and lipoids. Konopacka also considers these globules as a transition stage between 'primordial yolk' and yellow yolk.

Examining ovules at this stage of development, we have noted :

1. That there are great histochemical differences between primordial yolk and white yolk.
2. That white yolk-globules can be formed directly from material prolix in the ovule (therefore without first going through the primordial yolk form (Text-fig. 7)).
3. The appearance of the white yolk comes before and indicates the third stage of yolk-formation.
4. The appearance of these globules indicates a change in the metabolism of the ovule.

We do not deny from the morphological point of view the white yolk-globules represent a transition from primordial yolk to yellow yolk. But from the histochemical and histophysiological point of view, it seems to us that yellow yolk-globules are not derived from primordial yolk-globules. The latter, once formed, remain in this state until an advanced stage of incubation.<sup>1</sup>

<sup>1</sup> We do not believe in the transformation of primordial yolk into yellow yolk because:

(i) If the primordial yolk were to be changed into yellow yolk it would

Stage A is one of the most important of yolk-formation. During this stage the hen's egg changes its speed of growth as well as its metabolism. The hen's egg at the end of the second stage resembles, from the look of its yolk, holoblastic eggs (Riddle). During Stage A of the third phase of yolk-formation, the hen's egg undergoes changes which transform it from a holoblastic into a meroblastic ovule.

As we shall see later, the speed of the egg's growth, very slow during the second phase of yolk-formation, becomes much more rapid during Stage A of the third phase. At the same time, histochemically, the yolk changes considerably during this stage.

These changes leave their traces on the construction of the yolk. During Stage A these changes are manifested by a great variety of forms of vitelline globules. From the primordial yolk, rich in water and poor in fat and lipoids, a series of transitions occur in the yellow yolk, poor in water but very rich in fats and lipoids. In the fundamental substance of the globule, rich in water, the fats make their appearance in the form of inclusions of large diameter. These are the phospho-protein inclusions (Konopacka). The globules thus formed are the white yolk-globules.

Afterwards, according as the fundamental substance becomes enriched in fats (as demonstrated by Konopacka) and impoverished in water, the diameter of these inclusions diminishes until, in the yellow yolk-globule, these inclusions are seen no more except as very fine granulations.

The ovule which has changed its speed of growth accommodates itself little by little to this new metabolism. This accommodation is shown morphologically by the numerous forms of transition from primordial yolk to yellow yolk. These forms of transition are characteristic of Stage A of the third

disappear; the presence of primordial yolk inside the latebra argues against such change.

(ii) The primordial yolk represents 5-10 per cent. of the total yolk. Therefore it is practically impossible to conceive that this total of 5 per cent. of primordial yolk gives rise to the 95 per cent. of yellow yolk of the mature hen's egg.

(iii) Under the granular cortical layer one can see the appearance of white yolk-globules; these globules are formed without passing through the stage of primordial yolk (Text-fig. 7).

phase of yolk-formation. During Stage B of the same phase they no longer take place. But once formed the globules do not change. New globules formed later show a more advanced stage in the yolk-transformation up to the time when the yellow yolk is formed.

We have seen that the white yolk-globules can be formed at the periphery of the ovule without going through the stage of primordial yolk (Text-fig. 7).

Stage B of the Third Phase.—The appearance of the yellow yolk is preceded by a number of globules, which are the size of the yellow yolk-globules (50–100 microns), and some inclusions like white yolk. We have described these globules as ‘yolk of transition’. These globules have also been observed by Dubuisson and Konopacka. We have given them a particular name with the object of studying the histochemical construction of a stage of transition between the white and yellow yolk.

The appearance of the yellow yolk completely changes the structure of the ovule. It is formed in large quantity, and during this stage the growth of the ovule reaches its maximum. The thickness of the layers of yellow yolk is from 20 to 28 mm.

Stratification of the Yellow Yolk.—It is affirmed that the yellow yolk has a stratified appearance during this period.

Thompson in 1859 made a diagram, since become classical, wherein the yellow of the egg is represented in alternating layers of yellow and white yolk (the layers of yellow yolk being of greater thickness than those of the white (Diagram 5)). In a section of the yellow part of the egg boiled, it is frequently seen that some layers more yellow alternate with others less yellow.

But a certain number of authors criticize Thompson's diagram. Among the authors who doubt the stratification of the yolk are Balbiani, His, and, of more recent times, Dubuisson, van Durme, and Wasserman.

According to Balbiani, who has at some time studied the alternating of the yolk-layers, the difference lies in the presence or absence of yellow colouring matter. The arrangement of the yolk in concentric layers does not agree with reality, according to Dubuisson. Van Durme, in her diagram of the yolk

during the third phase of yolk-formation, does not show the yellow yolk stratified. Wasserman denies the existence of stratification in the yolk of the hen's egg. However, he has observed that vitelline elements are not always of the same size. One can distinguish smaller globular layers (round or polygonal) alternating with globular layers (polygonal or round) twice as big.

Our opinion on the stratification of the yolk is not entirely decided. We are about to repeat the experiments of Riddle and Gage on the stratification of the yolk, and the alternating of layers of white and yellow yolk.

### The Latebra.

Dubuisson and Konopacka have observed that the latebra is made up of several kinds of globules. But these authors have only given a very brief description of formation. We shall try to describe at greater length the different layers of globules which make up the latebra, and give a histophysiological explanation of the formation of this part of the hen's egg.

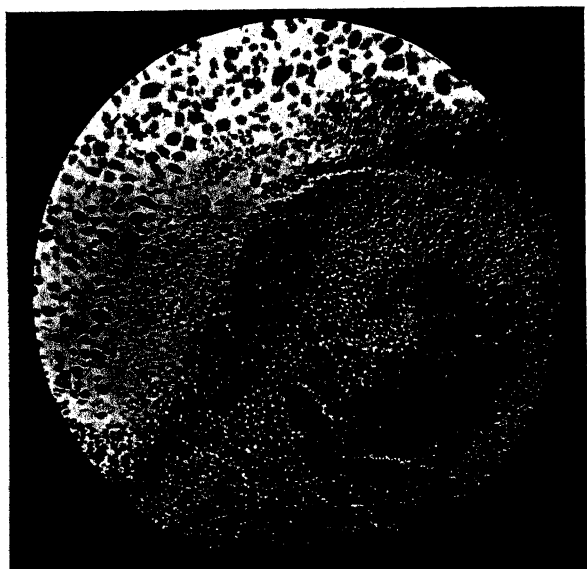
The latebra is made up of several layers of globules. In the centre these are very small (Text-figs. 10, A, and 11), and in appearance and size resemble the small globules of the endoplasmic zone (van Durme) (Stage B, second phase of yolk-formation). Outside this layer there are globules which in look, diameter, and reactions resemble the globules of primordial yolk of the exoplasmic zone (van Durme) (Stage B, second phase of yolk-formation) (Text-figs. 10, B, and 12). At the periphery there is a yolk-layer which resembles Konopacka's violet globules (Stage A of the third phase of yolk-formation), as well as Brambell's 'mitochondrial-cytoplasmic-yolk' and Dubuisson's yolk 'poor in fundamental substance'.

In our plates the position of this layer inside the latebra is indicated by the letter c (Text-fig. 10).

At the periphery of this layer there are globules of white yolk (Text-figs. 10, D, and 13). These are Konopacka's phosphoprotein globules. The layer of white yolk is the outermost of the latebra. It is continuous without any transition with the layers of yellow yolk (Text-fig. 10).



We have emphasized the structure of the latebra for two reasons: (1) because the succession of layers which constitute the latebra are only partially known; and (2) because we consider the latebra as made up of yolk formed at the time of the second phase of yolk-formation, yolk which has been compressed towards the centre of the ovule by layers of yellow yolk formed during the third phase.



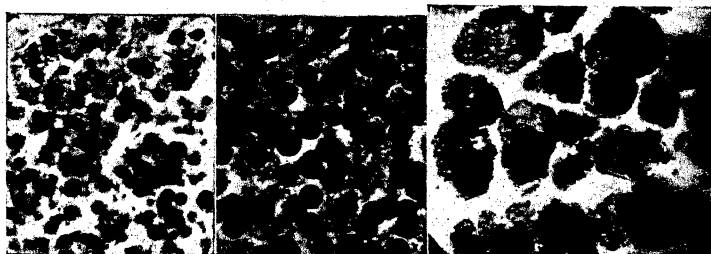
TEXT-FIG. 10. Latebra.

Ovary No. 74.—Fixative—alcohol 96. Stain haemaluncosin. Ovule of 20 mm. diameter ( $2\times A$ ). Whole view of latebra which is not homogeneous in construction. Four concentric layers clearly distinguishable. Outside latebra is a layer of transitional yolk and layers of yellow yolk (yy.).

As can be seen from Text-fig. 7, the diameter of the ovules at the end of the second phase of yolk-formation is 6 mm. It is also the diameter of the latebra.

Morphologically, one finds in the latebra all the layers which constitute the ovules of the second phase of yolk-formation:

(1) the small globules of primordial yolk (van Durme's endoplasmic zone); (2) the larger globules of primordial yolk (van Durme's exoplasmic zone); and (3) the white yolk-globules that are also found during Stage A of the third phase of yolk-formation. These are the exact layers which are also to be found in the latebra, with almost the same arrangement as in the yolk of ovules of 6 mm.



TEXT-FIG. 11.

TEXT-FIG. 12.

TEXT-FIG. 13.

Fig. 11.—Layer A of Fig. 10 ( $15\times D$ ), made up of very small globules of yolk (yolk of Durme's endoplasmic zone, second phase of yolk-formation).

Fig. 12.—Layer B of Fig. 10 ( $15\times D$ ), made up of larger globules of primordial yolk of Durme's exoplasmic zone (second phase of yolk-formation).

Fig. 13.—Layer D of Fig. 10 ( $15\times D$ ), made up of white yolk-globules, with small inclusions.

The central position of the latebra also shows an earlier formation, because new layers of yolk are always deposited at the periphery of the old layers. In this way the older layers are pushed back towards the centre of the ovule; the newly formed layers keep a more peripheral position.

Histochemically we have observed that the yolk-globules of the latebra are of the same composition as the ovules at the time of the second phase of yolk-formation.

Finally, to us the latebra is the yolk of the second phase of yolk-formation, pushed back deeper and deeper by newly formed layers of yellow yolk. The latebra draws away from the surface of the ovule, with which it has no further relation than as the intermediary of Pander's nucleus.

The latebra shows the hen's ovule with a holoblastic appearance. This conception nearly approaches Meckel's idea, which is that the latebra is the only formation of the hen's egg which corresponds to the mammalian egg.

According to Spohn and Riddle, the hen's egg during the second phase of yolk-formation has the appearance of a holoblastic egg like the eggs of many Vertebrata (fish, amphibians, &c.).

TABLE 3.

The relation between the diameter of ovules and the phases of yolk-formation of the hen's egg.

<i>Phases of Yolk-formation.</i>	<i>Appearance of the Ooplasm.</i>	<i>Diameter of the Ovules (in mm.).</i>
First Phase:	Balbani body	0.05-0.2
	Disaggregation of Balbani body	0.2-0.3
	Fat cortical layer	0.3-1.0
	Dispersion of fat-globules; cortical, vacuolar layer	1.0-2.0
Second Phase:		
Stage A . . .	Cortical granular layer; central vacu- olar layer	2.0-3.0
Stage B . . .	Cortical granular layer; primordial yolk (intravacuolar) layer	3.0-6.0
Third Phase:		
Stage A . . .	White yolk-layers; primordial yolk- layers; yolk of transition	6.0-9.0
Stage B . . .	White cortical yolk; yellow yolk; latebra; nucleus of Pander	10.0-35.0 <sup>1</sup>

During the third phase of yolk-formation the hen's egg goes a different way from these eggs. The great mass of yolk which is formed does not allow of total cleavage; the development of the hen's egg follows its own particular course.

The different phases of yolk-formation of the hen's egg represent as many stages of the phylogenetic evolution of birds. The first phase of yolk-formation is common to many Inverte-

<sup>1</sup> The diameter limits of the ovules in this column are not constant. They vary with the kind. In this table we have given the limits observed by us in sections made through the centre of the ovule.

brata and all Vertebrata. The second phase of yolk-formation of the hen's egg is the last stage of yolk-formation of many eggs of Vertebrata (certain kinds of fish, amphibians, &c., with holoblastic development). Finally, the third phase of yolk-formation is only to be seen in eggs of certain fish ('Ganoids' and Selachii), reptiles, and birds.

The majority of these forms do not seem to produce yellow yolk. They would stop at Stage A of the third phase of yolk-formation of the hen's egg.

### Intra-ovular Organelles.

During the first phase of yolk-formation only very small differences exist between the behaviour of the intra-ovular organelles in the hen's egg and in the eggs of other Vertebrata and many Invertebrata. In the present work we shall give a brief summary of the evolution of these organelles during yolk-formation of the hen's egg.

#### Mitochondria.

Mitochondria are in very small number in the ooplasm of the oogonia (d'Hollander). Their number becomes much greater in oocytes with Balbiani body. At the periphery of this formation (in what is called the 'yolk-forming layer') there is a large mitochondrial accumulation.

When the ovules are 200 microns in diameter the Balbiani body begins to disintegrate. Mitochondria are then distributed into the whole of the ooplasm. A certain number remain at the periphery of the ovule where they constitute a layer, the mitochondrial cortical layer (van Durme).

There has also been described a mitochondrial, perinuclear layer, discontinuous, however, which disappears during the ovule's development (Durme, Brambell, R. S. Das).

At the time of the second phase of yolk-formation the mitochondria once again form the mitochondrial cortical layer. Other mitochondria are distributed in the intervacuolar reticulum of the central region of the ovule (van Durme).

In larger ovules the mitochondria have not been examined,

the attention of morphologists being more taken up with changes in place and form of these organelles at the beginning of the first phase of yolk-formation.

The chondriome is mainly made up of chondriocents (van Durme).

We have seen that the number of mitochondria is increased considerably from the Balbiani body up to the appearance of the primordial yolk. This multiplication has been explained in different ways. It seems to us that the most plausible is that based on the external material brought from the substances which contribute to the building up of the mitochondria.

In a series of experiments made from 1906 to 1912, Russo has demonstrated that by injecting lecithin into the peritoneum of a female rabbit, it is first seen again in the germinal epithelium covering the ovary, afterwards in the follicular epithelium, the zona radiata, and the ooplasm.

Several years later Bhattacharya (on the tortoise's egg) and Das (on the hen's egg) have noted the passage of mitochondria from the follicular epithelium to the ovule.

### Golgi Apparatus.

In the same manner as the mitochondria Golgi elements are very reduced in number in the oogonia (Berenberg-Gossler and Woodger). Their number increases in ovules which have the Balbiani body (Brambell).

In the majority of kinds of ovules at this stage mitochondria are to be found intermingled with Golgi elements. In the hen's egg Golgi elements are found at the periphery of the Balbiani body, according to Brambell.

After the disintegration of the Balbiani body the Golgi elements are also scattered, and follow an arrangement similar to that of the mitochondria. They constitute two layers, the one cortical, the other perinuclear.

In larger ovules the Golgi elements begin to break up and lose their affinity for osmium and silver (Brambell). The number of Golgi elements, reduced to two or three in the ooplasm of the oogonia, greatly increases afterwards.

Brambell (1925), Bhattacharya (1925), and Das (1931) have

remarked the passage of Golgi elements from the follicular epithelium to the ovule.

From what we have shown above, it can be seen that in certain cases one can discern by morphological methods the passage of substances from the ovule membranes to the ooplasm. But in the majority of cases the substances which pass through the follicular epithelium are of a fluid nature, and are of such composition as is not detectable by actual morphological methods.

The great number of mitochondria in one cell signifies an intense intracellular metabolism (Cowdry and Champy); the mitochondria are intracellular stages which by their number increase the surface cytoplasmic exchange, and which, due to their chemical construction, interfere in the intracellular synthesis.

Judging from the great number of mitochondria and Golgi elements that are seen during the first stage of yolk-formation, it can be supposed that the substances which have penetrated into the ooplasm are transformed by the influence of intravovular organelles. The histochemical construction of the organelles of the ovule leads one to believe that they interfere in the molecular changes of the ooplasm.

Mitochondria are lipid-protein complexes. Around the vacuoles of the Golgi apparatus there are also lipoids. Among these lipoids (mitochondrial and Golgi) lecithin prevails. According to Fauré-Fremiet, Mayer, and Schaeffer, the lecithin, by the unsaturated fatty acids that it possesses, would play an important part in the phenomena of intramolecular oxidation-reduction.

Recently Voss has demonstrated the existence of oxidases in the region of the vitelline nucleus of Balbiani in the frog's egg.

Konopacka, studying the hen's egg, has found that the fat substances undergo important histochemical changes during the first phase of yolk-formation.

Guthrie denies the part of the mitochondria in the formation of yolk, but she is of the opinion that the organelles interfere in the chemical changes of the ooplasm. It is also our own opinion in the matter.

We think that these organelles do not change into yolk. They do the part of catalizing the chemical changes of the ooplasm, and preparing the latter for yolk-formation proper, which only begins at a later stage.

#### SPEED OF GROWTH IN THE HEN'S OOCYTE.

The ovule does not grow with the same speed from its differentiation up to the end of the third phase of yolk-formation.

Different authors who have studied the speed of growth of the hen's egg have distinguished two periods: the first very slow, the second very fast. The period of slow growth begins as soon as the differentiation of the first-order oocytes (fifteenth day of incubation), and finishes, according to Riddle, when the ovules have reached a 6-mm. diameter. The period of quick growth begins as soon as the ovules have surpassed the 6-mm. diameter, according to Riddle, or the 9-mm. diameter, according to Stieve.

#### Slow Development.

The period of slow development can last from several months to several years. The growth of the ovules is not continuous; periods of growth are followed by periods of rest (d'Hollander, Sonnenbrodt, Fauré-Fremiet, and Kaufmann). Moreover, this growth does not affect all ovules, which explains why certain ovules evolve quicker than others.

The period of slow growth can be subdivided into several stages. The first stage begins during the extra-follicular period of the oocyte (therefore during incubation), and continues after hatching up to when the ovules have reached 60-80 microns diameter (d'Hollander, Sonnenbrodt, Barthelmez, Fauré-Fremiet and Kaufmann, Brambell).

Having reached 80 microns in diameter the ovule's growth seems to stop or to relax for a space of time which can vary from 11 weeks (Brambell) to several years.

In the pigeon's egg Barthelmez describes a period of growth from 80 to 100 microns to 400 microns in diameter. Of the growth of the hen's egg from 80 microns to 3 mm. diameter very little is known. At 3 mm. diameter the ovule pediculates

(Riddle, Stieve). Certain authors badly define all these stages as one single period of growth, that of ovules below 3 mm. diameter (Romanoff).

From 3 to 6 mm. diameter the ovule enters upon a period of growth which is better known. During this period the ovules grow twenty-five times less quickly than during the quick period of growth (Riddle), which would make a growth of 80 microns a day, and a minimum duration of 60 days, always supposing that during this time the increase in diameter were regular and continuous.

### Quick Development.

The disagreement which exists between Riddle and Stieve as regards the diameter of ovules at the beginning of the quick-growth period can be explained thus: Riddle has remarked in ovules which have passed 6 mm. diameter a stratified appearance of yolk. In an ovule 7 mm. diameter he has noted layers of yolk of 0.250 mm. thickness, while in larger ovules the thickness of the yolk-layers formed during a day is 2 mm.

Stieve, in his turn, says that an ovule has entered upon its quick-growth period when it changes its colour and begins to form yellow yolk. That is to say, ovules do not form yellow yolk until they have passed Stage A of the third phase of yolk-formation, which is 9-10 mm. diameter.

Stage A of Quick Growth.—Stieve most often finds growth less quick at the beginning of the period of quick development in the hen's egg. In the majority of graphs of his work it is very clearly seen that the curve of development of the mass of ovules presents a much weaker gradient at the beginning of the quick-growth period, and a very decided gradient during the rest of this period (see Diagrams 1 and 2, curve for the mass of ovules).

This relaxed growth in eggs from 6 to 9 mm. diameter we have called Stage A of the quick-growth period. Ovules during this stage present the appearance, morphologically and histo-chemically, of ovules during Stage A of the third phase of yolk-formation. It therefore comes about that there is an almost complete correlation between the morphological aspect and the



speed of the ovule's growth during yolk-formation. The first and second phases of yolk-formation correspond to the period of slow growth; the third phase of yolk-formation to the period of quick growth, stage by stage.

Stage A of quick growth does not last long. The minimum

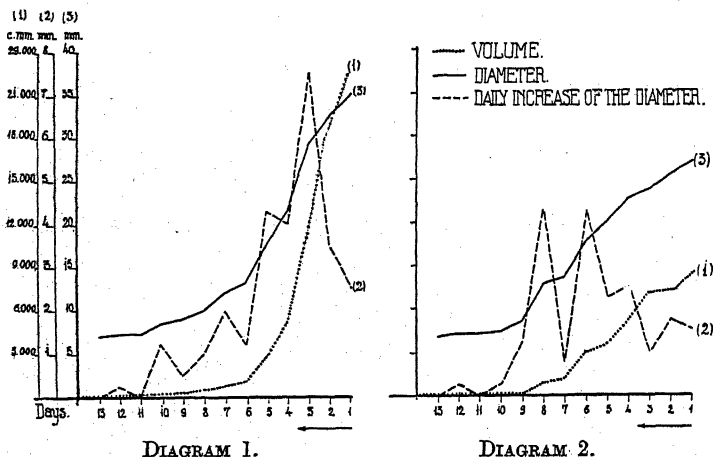


DIAGRAM 1.

DIAGRAM 2.

The variations in volume and diameter of the hen's egg during the last days of growth (according to Stieve's data). Curve 1 represents the variations in volume of the ovules; curve 2 the daily development of the diameter during growth. Curves 1 and 3 show: (a) a slower rate of growth (Stage A of the period of quick growth), and (b) another rate where the curve rises very quickly (Stage B of this period). These two stages are better seen in Diagram 1. Curve 2 shows that the diameter of ovules does not increase regularly. As a general rule it is seen that, if during 1 day the ovule has increased more quickly in diameter, the following day its increase is relaxed. Diagram 1 represents Hen No. 4, which laid every day. Diagram 2, Hen No. 13, which only laid once every 2 days (Table 4).

time is 1 day, the maximum 5 days. It can be lacking altogether, but this absence is extremely rare.

It follows from Stieve's tables that during this stage the ovule increases from 0.25 to 0.5 mm. diameter per day. This is also the width of the layers found by Riddle in the ovule of 7 mm. Stieve does not specify the diameter which ovules show at the beginning of Stage A. In the majority of ovaries examined

by him this stage begins when the ovules have reached 7-7.5 mm. diameter.

Histochemically, we have fixed the limits of this stage between 6 and 9 mm. These limits can vary with the breed, size, &c.

**Stage B of Quick Growth.**—Ovules which have passed 9-10 mm. diameter grow very quickly in volume and diameter. It is Stage B of the ovule's quick-growth period. The duration of this stage is from 5 to 9 days. At this time the yellow yolk makes its appearance.

For this stage Riddle gives us the following characteristics: (1) The swift change in the speed of growth. In the space of a single day the ovule passes from a rather slow development to a coefficient of growth from eight to twenty times greater. (2) Growth would be on an average twenty-five times quicker than during slow-growth period. (3) Ovules grow by 2 mm. a day.

Stieve has shown that at this stage of growth the quantity of yolk deposited each day in the ovule is subject to great variation. As a rule it will be seen that, if during one day a greater quantity of yolk is deposited, the following day the quantity is less great.

The variations in volume in the first place reflect on the increase in diameter in ovules. The quantity of yolk deposited each day, being subject to variation, the daily increase in diameter undergoes great variation (see Table 4, and curve 2 of Diagrams 1 and 2). The variation in diameter shows ups and downs from one day to the next. It is therefore seen that during Stage B growth is not regular.

To sum up: from the differentiation up to ejection the oocyte goes through two periods of growth: one very long, the other very short. During the first phase the speed of growth is much reduced; during the second phase ovules grow very quickly both in volume and diameter.

The time necessary to pass through these periods is not very well known for the period of slow development. We are better informed as regards the quick period of growth of the hen's egg.

As a rule there is a concordance between the speed of growth and the phases of yolk-formation.

TABLE 4.

The increase in volume and diameter of the eggs of Hens Nos. 4 and 13 during the quick period of growth. Of these two, Hen No. 4 lays every day; No. 13 every other day (data supplied by Stieve).

<i>Last Days of Growth.</i>	<i>Hen No. 4.</i>			<i>Hen No. 13.</i>		
	<i>Diameter of Ovules (in mm.).</i>	<i>Daily Development of Diameter.</i>	<i>Volume in mm<sup>3</sup>.</i>	<i>Diameter of Ovules (in mm.).</i>	<i>Daily Development of Diameter.</i>	<i>Volume in mm<sup>3</sup>.</i>
13	7.0	0.0	..	6.75	..	..
12	7.25	0.25	..	6.75	0.0	..
11	7.25	0.0	..	7.0	0.25	175
10	8.5	1.25	321.5	7.0	0.0	175
9	9.0	0.5	381.7	7.25	0.25	190
8	10.0	1.0	523.5	12.75	5.5	1,080
7	12.0	2.0	904.6	13.5	0.75	1,288
6	13.2	1.2	1,204.0	18.0	4.5	3,053
5	17.5	4.3	2,806.0	19.75	1.75	3,734
4	21.5	4.0	5,203.0	21.75	2.0	5,379
3	29.0	7.5	12,770.0	23.75	2.0	7,004
2	32.5	3.5	17,970.0	24.75	1.0	7,398
1	35.0	2.5	22,445.0	25.25	0.75	8,680
Laying	..	..	..	..	..	..

## II. THE FOLLICULAR EPITHELIUM DURING GROWTH

### INTRODUCTION.

The hen's egg has several membranes: the external theca, the internal theca (Pearl and Boring; Das), and the follicular epithelium.

Among the ovule membranes the follicular epithelium plays an important part, which has decided us to devote a separate chapter to it. This membrane is very intimately connected with the ovule; it forms a continuous covering for it, which divides the ovule from the rest of the ovary. This characteristic is not peculiar to hens' ovules, but is found in the case of the majority of Invertebrata and of all Vertebrata eggs.

This membrane does the work of an ultrafilter, of which the

chemical composition and the permeability change several times during the ovule's growth.

For all these reasons we consider that the changes observed at the level of the follicular epithelium during yolk-formation deserve special description.

#### THE FOLLICULAR EPITHELIUM DURING THE OVULE'S DEVELOPMENT.

##### Differentiation.

In the hen's egg the follicular cells make their appearance during the sixteenth day of incubation, but they only begin to surround the ovules 10 days later, that is to say, 5 days after hatching (d'Hollander).

At this time the follicular cells are very flat, and not numerous, but they form a continuous covering to the ovule; by this detail the hen's egg is distinguished from the batrachian egg, where the follicular epithelium forms a discontinuous covering at the beginning of its growth (Schultze; Konopacki).

##### Variations in Depth of Follicular Cells.

In a general way variations in depth of follicular cells have been shown for the hen's egg by Loyez (1906), Brambell (1925), Das, and V. Marza, E. Marza and Chiosa (1932). But up to now no strict demonstration exists of the ratio between dimensions of follicular cells and ooplasmic changes.

It has been seen that the follicular cells increase in number and depth during the first phase of yolk-formation. It is admitted that the follicular epithelium reaches a maximum depth, and even that it becomes pluristratified during the second phase of yolk-formation (Loyez, Brambell, Das), and that towards the end of the ovule's growth (therefore towards the end of the third phase) the depth of the follicular epithelium decreases very much.

During our work on the hen's egg we have discovered that a number of these affirmations were open to criticism, above all those relative to the stratification of the follicular epithelium, and to the relation between depths of follicular cells and phases of yolk-formation.

To dispel the uncertainty left by Loyez, Brambell, and Das in their works, we have studied the variations in depth of the follicular cells at the time of the three phases of yolk-formation.

We have therefore studied 1,840 ovules, of which 1,531 were during their first phase of yolk-formation, 262 during their second phase, and 47 during the last phase. The diameters of each ovule were measured and the depth of the follicular epithelium at three or four points of its circumference. For each group of ovules we have calculated the average depth of the epithelium. The results obtained we have shown in Table 5. In this table, next to the average depth of the follicular epithelium, two columns are devoted to its maximum and minimum depth.

It is necessary to say, at this point, that the depth of the follicular cells varies very much in different parts of the same ovule, but by multiplying the measurements and avoiding oblique sections we have established in each ovule the average depth of its epithelium; and one by one, afterwards, we have established the average depth of follicular cells in each group of examined ovules. In this way the number of examinations made on these ovules is much greater than the number of ovules examined in each group.

In Table 5 and Diagram 3 and Text-figs. 14-17, it is seen that, during the first phase of yolk-formation, the follicular epithelium increases continually in depth. The follicular cells, very flat at the beginning of this phase (Text-fig. 14), become very deep at the end of this phase (Diagram 3).

During Stage A of the second phase the follicular epithelium maintains its depth, but during Stage B this depth begins to diminish. This diminution is fairly swift (Diagram 3). The ovules of 6 mm. diameter have only, on an average, 7.1 microns in depth.

During the third phase of yolk-formation the depth continues to go down up to the end of this period, when the follicular cells are no more than 3.6 microns deep (Diagram 3 and Text-fig. 20).

In the first part of this work we have seen that the yolk makes its appearance in ovules of 3 mm. diameter and continues

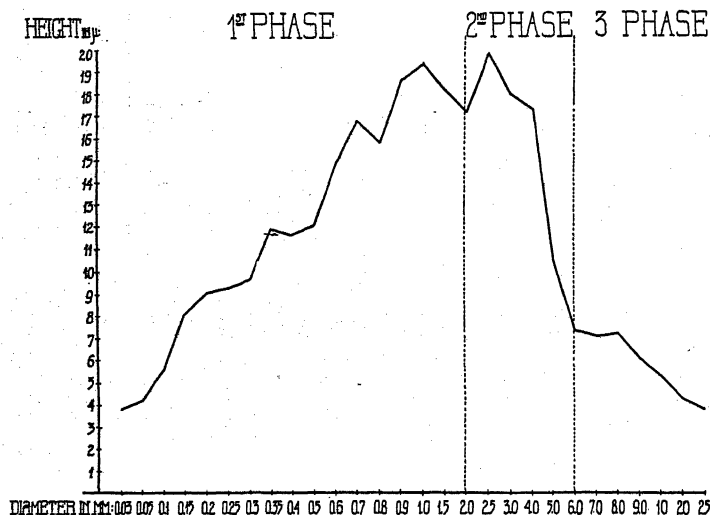
TABLE 5.

The depth of the follicular epithelium during the three phases of  
yolk-formation of the hen's egg.

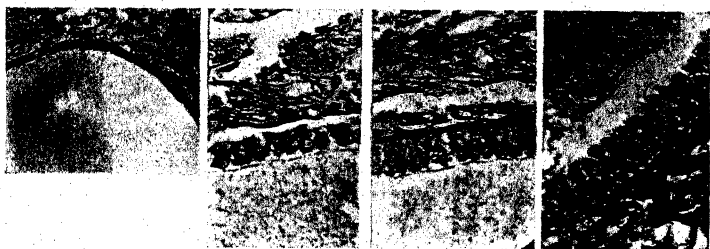
<i>Diameter of Ovules (in mm.).</i>	<i>Depth of Follicular Epithelium in Microns.</i>			<i>Number of Ovules Examined.</i>
	<i>Average.</i>	<i>Maximum.</i>	<i>Minimum.</i>	
First Phase:				
0.03	3.8	4.0	3.3	7
0.05	4.2	4.5	3.8	87
0.1	5.6	8.0	4.4	297
0.15	8.1	11.0	5.3	87
0.2	9.0	12.0	7.3	216
0.25	9.3	13.0	7.2	302
0.3	9.6	13.0	8.2	142
0.35	11.7	15.0	10.0	57
0.4	12.2	17.0	11.0	60
0.5	12.0	17.0	10.7	86
0.6	14.7	21.0	11.7	82
0.7	16.7	23.0	12.0	54
0.8	15.7	21.0	14.4	33
0.9	18.5	21.0	14.4	16
1.0	19.3	25.0	13.0	23
1.5	19.0	30.0	17.0	57
				1,531
Second Phase:				
2.0	17.1	33.0	13.0	49
2.5	19.7	33.0	14.0	50
3.0	17.8	31.0	17.0	17
4.0	17.1	29.0	12.0	49
5.0	10.3	16.0	6.0	16
6.0	7.1	12.0	5.8	14
				262
Third Phase:				
7.0	6.9	9.0	4.7	7
8.0	7.0	10.0	4.5	8
9.0	5.9	6.0	4.2	7
10.0	5.1	6.0	4.2	15
20.0	4.1	8.0	3.3	16
25.0	3.6	4.1	3.3	9
				47
			Total	1,840

afterwards up to the end of the third phase. Therefore, during all this time the depth of the follicular epithelium diminishes continually. An inverse ratio exists between yolk-formation

and the depth of cells. In the hen's egg the beginning of the precipitation of yolk into the ovule coincides with the beginning of the diminution of depth in the follicular cells. During the third phase, when 90 per cent. of the yolk which will constitute the deutoplasm of the mature egg is deposited into the ovule, their depth is very reduced (Text-fig. 20).



Yet this ovule, at the inside of which the yolk has not made its appearance, measures 2.5–2.8 mm. in diameter. And there are many more examples of ovules where precipitation of the yolk only begins in ovules having approximately 3 mm. diameter.



TEXT-FIGS. 14–17.

TEXT-FIGS. 14–17. Variations in Depth of the Follicular Epithelium.

Fig. 14.—Ovary No. 75. Fixative—alcohol 96. Acid proteins. Ovule of  $176 \times 235$  microns diameter ( $6 \times D$ ). Follicular epithelium made up of flat cubical cells of 3.3 microns depth.

Fig. 15.—Ovary No. 24. Fixative—alcohol 96. Acid proteins. Ovule of  $400 \times 644$  microns diameter ( $6 \times D$ ). Depth of follicular epithelium is 12.6 microns. Just as in preceding figure, ovoplasm very poor in acid proteins.

Fig. 16.—Ovary No. 24. Fixative—alcohol 96. Acid proteins. Ovule of  $900 \times 1,080$  microns diameter ( $6 \times D$ ). Depth of follicular epithelium is 21 microns. Radiated zone has not yet appeared. Ovoplasm, in its granular appearance, also very poor in acid proteins, in comparison with ovule membranes.

Fig. 17.—Ovary No. 7. Fixative—bichromate of potassium; stain ferric haematoxylin. Ovule of  $1,000 \times 1,200$  microns diameter ( $6 \times D$ ). Depth of follicular epithelium is 25.2 microns. Nuclei of cells in two layers. Radiated zone has not yet appeared.

What is interesting to note is that at the end of the first phase and during Stage A of the second phase (therefore during the period when the transparent vacuoles become very numerous in the ooplasm) the depth of the follicular epithelium is particularly great.

In ovules where the follicular epithelium reaches the maximum of its development, Loyez, Brambell, and Das state that it



is pluristratified, and that it can contain up to five cellular layers.

Examining the follicular epithelium we have found that it is never pluristratified, but pseudostratified (Text-figs. 16, 17, and 18). The nuclei are placed at two different levels, but it is



TEXT-FIGS. 18-20.

Fig. 18.—Ovary No. 7. Fixative—bichromate of potassium; stain ferric haematoxylin. Ovule of  $3.2 \times 4.5$  mm. diameter (second phase of yolk-formation) ( $6 \times D$ ). Depth of follicular epithelium is 29 microns. Pseudo-stratified placing of follicular epithelium is better seen than in Figs. 15, 16, and 17. Cells arranged in a single layer; nuclei at varying depths. Follicular epithelium detached from the ovoplasm. Radiated zone not visible.

Fig. 19.—Ovary No. 77. Fixative—alcohol 96. Acid proteins. Ovule of  $5 \times 6.5$  mm. diameter ( $6 \times D$ ). Very few structural details are seen of intensely coloured follicular epithelium. Its depth is 19 microns. Radiated zone appears, hardly perceptible, and poor in acid proteins; granular cortical layer, and a few globules of yolk in vacuoles.

Fig. 20.—Ovary 74. Fixative—alcohol 96. Acid proteins. Ovule of 20 mm. diameter ( $6 \times D$ ). Internal theca is richer in acid proteins than juxta-epithelial layer (2). Blood capillaries in both (c.). Follicular epithelium of flat cubic cells, of 3.3 microns depth. A few globules of yolk hardly visible.

easily seen that these cells begin at the radiated zone and finish at the internal theca without presenting a pluristratified appearance (Text-fig. 18). Only Dubuissou has described the nuclei at all well, which, according to him, form 'two layers, more often alternating than not'. Other writers have examined sections made in the thickness of the follicular epithelium, sections which give it a pluristratified appearance.

### The Two Varieties in Cells of the Follicular Epithelium.

In the follicular epithelium of birds' eggs Holl has observed two kinds of cells. The one 'Schutzzellen' take colours intensely (chromophil cells), others remain transparent; the latter, according to Holl, would play a nutritive part ('Nahrzellen'). Mertens confirms Holl's observations. A little later Loyez (1906) denies the existence of these two varieties of cells.

They are considered by Loyez as artefacts, due to the fixation and retraction of the tissues. More recently still Brambell comes up against these affirmations of Loyez. He finds in the hen's egg the two varieties of cells described by Holl. According to him the chromophil cells are derived from the transparent cells. By the help of mitochondrial fixatives Das has also observed Holl's two varieties.

According to Brambell the two varieties are only observed in ovules during the second phase of yolk-formation. In a subsequent phase the chromophil cells are transformed into inter-cellular cement, which takes colouring intensely, and which would be of the same nature as the radiations of the radiated zone.

With fixatives used for the detection of cholesterol and proteins (formol, alcohol, sublimate, &c.) we have never observed these two cellular varieties, but we have observed them after the use of bichromate.

### Chondriome, Golgi Apparatus.

#### Organelles.

Loyez, examining the chondriome of the follicular cell, has noted the displacement of these organelles during yolk-formation. Brambell and Das describe a granulous chondriome in the follicular cells spread over the whole cytoplasm (Brambell) or accumulated round the nucleus (Das). Russo, in a series of experiments made on the rabbit's egg, Bhattacharya on the tortoise's egg, and Das on the hen's egg, have noted the passage of mitochondria from the follicular epithelium to the ovule.

The Golgi apparatus of the follicular cells is situated next

to the nucleus, or between it and the surface of the oocyte (Brambell). The Golgi apparatus is spherical or elongated, and a little smaller than the nuclei of the follicular cells (Brambell, Das). The two last authors, studying the hen's egg, and Bhattacharya, studying the tortoise's egg, have noted the passage of Golgi elements from the follicular epithelium to the ooplasm.

These observations constitute so many morphological proofs of a form of passage of the substances which impregnate the ovular membranes to the ooplasm.

### Chemistry of the Follicular Epithelium.

From the chemical point of view, the follicular epithelium of the hen's egg has been very little studied. Since the work of Riddle and Lawrence (1915) there has been no further analysis of this membrane.

Riddle and Lawrence have separated the 'follicular membrane' of the internal theca, which is particularly difficult to do. They have analysed, in these membranes, the proteins, fats, lipids, water, ash, and extractive substances.

Riddle and Lawrence have examined membranes of ovules of: 3 to 5 mm. (experiment *a*); from 4 to 6 mm. (experiment *b*); from 5 to 13 mm. (experiment *c*); from 15 to 30 mm. (experiment *d*); and larger ovules (experiment *dd*). A part of these results have already been shown when dealing with the chemistry of yolk-formation (see Tables 9, 10, 11, and 14, and Diagrams 6 and 7).

Riddle and Lawrence have established that the follicular epithelium of ovules of 3-5 mm. diameter are poorer in P lipoidic than ovules of 5-6 mm. diameter. These latter are very rich in phosphatids and very poor in neutral fats. In comparison with what one finds in ovules of 3-5 mm. diameter the ratio phosphatids/neutral fats is completely reversed in the follicular epithelium of ovules from 5 to 6 mm. diameter. In the follicular epithelium of larger ovules this ratio returns to its initial value (see Table 10 and Diagram 7).

The proteins (Table 11) and membrane water (Table 9 and Diagram 6) only vary very little during the two last stages of yolk-formation.

### Histochemistry of the Follicular Epithelium.

Histochemically, Marza and Marza have noted that the follicular epithelium is very poor in cholesterol during the first and second phase of yolk-formation. It is invaded with cholesterol during the third stage of yolk-formation (Table 6). Moreover, the cholesterol that one finds in the follicular epithelium does not belong to cells of this membrane; it represents only a phase of passage of this substance from the internal theca to the ooplasm. In this way Marza and Marza explain the variations in cholesterol of the different regions of the follicular epithelium of the same ovule.

TABLE 6.

Membranes of ovules. Average number of cholesterol crystals in the standard unity of surface (0.10 mm<sup>2</sup>).

<i>Phase of Yolk-formation.</i>	<i>Diameter of Ovules in mm.</i>	<i>Internal Theca.</i>	<i>Juxta-epithelial Layer.</i>	<i>Follicular Epithelium.</i>	<i>Radiated Zone.</i>
First Phase:	0.05	19.3	22.3	13.9	..
	0.1	25.5	50.1	17.3	26.8
	0.3	27.9	44.0	11.3	31.2
	0.6	26.9	10.1	11.4	16.9
Second Phase:	2.0	20.9	23.3	7.0	20.0
	6.0	37.2	59.0	27.7	50.4
Third Phase:	15.0	590.0	140.0	132.0	85.0

In the same manner must be interpreted Margaret Murray's observations on the grasshopper's egg. In the follicular epithelium of this insect she finds cells rich in chondriome by the side of cells poor in chondriome. Murray supposes that the differences are due to waves of protoplasmic activity in cells of the follicular epithelium.

In the hen's egg we have shown that the diffusion of cholesterol towards the ovule is also in waves, and that one can seize upon all the phases of impregnation of the ovular membranes (follicular theca, juxta-epithelial layer, follicular epithelium, radiated zone) by the cholesterol.

The follicular cells of amphibians enclose very fine lipoidic grains (Ciaccio) of which the localization undergoes a fluctuation

in proportion to the maturity of the ovule. On the same material, Konopacki, Konopacki and Konopacka have shown that the follicular epithelium of ovules below 200 microns diameter are filled with lipoidic granulations. In ovules which have passed beyond this diameter one observes very important histochemical changes in relation to the appearance of the radiated zone.

Iron is absent from the follicular epithelium cells (Marza, Marza and Chiosa).

In the follicular epithelium ash is more abundant at the pole in relation to the internal theca (second phase). During the first and third phase the cells are a little less rich in ash than during the second phase of yolk-formation (Policard's method of micro-incineration).

By this method it is seen that the nuclei of follicular cells do not possess fixed ash (Ca, Mg, Fe, Si, P) contrary to what one sees in all other nuclei of the hen's ovary (Marza, Marza and Chiosa).

The nuclei of follicular cells show an intense nuclear reaction (Feulgen's method), while the nucleus of the oocyte shows a negative reaction when the ovule has passed 50-80 microns diameter (Marza and Marza, 1934).

### Histophysiology.

From the histophysiological point of view the follicular epithelium has been considered as an intermediary between the organism and the ovule, in turn active or passive.

According to many authors the cells of the follicular epithelium interfere actively in the transformation of the substances which penetrate into the oocyte. The yolk which is formed in the peripheral layers of the ovule would be elaborated by follicular cells.

Another group of authors considers the follicular epithelium as a living membrane, which from its chemical construction facilitates the transport of nutritive substances into the egg. Among the authors of this second group there are Riddle, Spohn, Konopacki, Konopacki and Konopacka, Bialaszewicz, M. Murray; and Russo, Brambell, Bhattacharya, and Das who

have observed the direct passage of the morphological elements from the follicular epithelium to the ooplasm.

In the cultivation *in vitro* of the follicular cells of the grasshopper's egg, Murray has observed the very swift passage of distal small drops from the follicular cells to the centre of cultivation (inversely to the passage of the substances which give birth to the yolk). Furthermore, Murray has demonstrated that the follicular cells do not all function with equal intensity. By the side of cells showing very evident signs of functioning one finds cells functioning to a lesser degree.

In the frog's egg Konopacki and Konopacka have studied variations in form and histochemical construction of the follicular epithelium during yolk-formation. They have come to the conclusion that it is an intermediary between the organism and the ovule.

Riddle, Spohn and Riddle, studying rapidity of growth in the hen's egg, find that the speed in the formation of yolk is so great during the last phase of yolk-formation that it is necessary that a follicular cell be capable of secreting every half-hour a quantity of yolk equal to its volume, which is not possible. Therefore, according to them, the follicular epithelium does not interfere in the elaboration of yolk-substances.

By ultrafiltration Bialaszewicz has shown that the yolk has a thermionic composition very different from that of the blood-plasm from which it is derived. Egg yolk (of Invertebrata and Vertebrata) is very rich in potassium and very poor in sodium. It results that the cells of the follicular epithelium play the part of a selective ultrafilter.

In our opinion the hypothesis of an elaboration of the yolk by follicular cells is hardly likely. If the high aspect of the cells (during certain phases of the ovule's evolution) and the richness in chondriome and Golgi elements can give rise to such hypotheses, the aspect of the same cells during the third phase of yolk-formation leaves no doubt in this respect. During this phase the cells are very reduced in depth and have no secretory aspect; and yet during this period the greatest part of the yolk is formed. If the follicular epithelium interferes in yolk-formation, its part is reduced to that of selecting the material which

crosses it. In this function the follicular epithelium shows variations in relation to changes in chemical construction and the electric polarization of the cytoplasmic cortical film of these cells during the different stages of yolk-formation.

### III. HISTOCHEMISTRY OF YOLK-FORMATION

#### Fats and Lipoids.

The distribution of fats in the hen's egg has been described by E. de Sommer (1905); M. Loyez (1906); M. van Durme (1914); Brambell (1925); Das (1931); and Konopacka (1931 and 1933).

**First Phase of Yolk-Formation.**—This phase is characterized by the accumulation in the ooplasm of a great quantity of fats, colourable Sudan III, Nile blue, osmic acid, &c. This accumulation of fat substances has been remarked in the ovules of all Vertebrata and many Invertebrata.

The fat substances once in the ooplasm take various aspects. Some (the unsaturated fatty acids) contribute to the building up of mitochondria and Golgi apparatus; others (above all the saturated acids) form fat-balls; a third lot form lipoproteins with the continuous phase of ooplasm.

Lipoids have been found near the Balbiani body (Ciaccio, Parat). The neutral fats are localized in the form of balls in the immediate neighbourhood of the Balbiani body. Altogether these balls form the 'fatty cap' which covers the nucleus of the ovule (E. de Sommer, Loyez, van Durme, Konopacka).

In a short time the balls of fat fill up the whole ooplasm, as Konopacka and we ourselves have remarked at the time of our study of the cholesterol (ovules of 100–200 microns diameter).

In larger ovules the balls of fat constitute a peripheral layer (Durme's fat granular layer). This layer is situated on the inside of the cortical mitochondrial layer (Text-fig. 2).

One can also find a second layer of fat around the nucleus (Durme's perinuclear fat granular layer).

According to Konopacka the fats which appear in the young ovules (beginning of first phase of yolk-formation) change constantly chemically. The fats of the 'fatty cap' (Balbiani

body ovules) are made up of phosphatids. In larger ovules phosphatids form only a surface to the ball of fat, the centre being made up of neutral fats.

After disaggregation of the Balbiani body, and the formation of the fat granular peripheral layer, the fat-balls are entirely made up of neutral fats (ovules from 300 to 900 microns diameter).

**Second Phase of Yolk-formation.**—At the periphery of the ovule the fat granular cortical layer is still seen. In the central vacuolar layer the fat is found in the form of small drops in the thick part of the intervacuolar reticulum.

The primordial yolk-globules would contain fats as well (Loyez, Konopacka), which, in combining with the proteins, make the yolk-globule insoluble in fat solvents.

**Third Phase of Yolk-formation.**—The forms of transition from primordial yolk to yellow yolk contain various quantities of fats and lipoids. Konopacka has shown the phases of the invasion of fats in these forms very well. The white yolk-globules are much richer in fats than the primordial yolk-globules. The fat is at first localized in the phosphoprotein globules; the fundamental substance of the globule shows only a few fats. In the most evolved forms the fats invade the fundamental substance. Finally, in the yellow yolk-globules only neutral fats are seen. The lipoids (lecithin) do not react to iodine, according to Romieu's method. The presence of lecithin is no longer detectable because of the chemical connexion between this substance and vitellin.

Marza (1929) has shown that in the parablastic region where, under the action of cicatricular enzymes, the large chemical molecules are divided and the yolk-globules crumble, lecithin is once again detectable histochemically.

In the latebra, on the surface of the phosphoprotein globules,<sup>1</sup> Konopacka finds phosphatides and neutral fats slightly colourable with Sudan III.

To sum up: during the first phase of yolk-formation an abundance of fats is seen in the ooplasm; during the second phase the quantity of fats in the ooplasm is much less; becoming again greater during the third phase.

<sup>1</sup> The layer 'D' of the latebra, according to our conception.



### Cholesterol.

The cholesterol has been well studied chemically in the mature hen's egg. The histochemical detection of cholesterol has only been incompletely performed.

It was not until 1932 that V. Marza and E. Marza showed the histochemical localization of cholesterol during the whole of the process of yolk-formation of the hen's egg. These writers used Leulier and Noel's method.

Marza and Marza counted the number of refringent crystals in the ovule membranes and in the ooplasm, relating this number to a standard surface.

In the case of the fat-balls or yolk-globules, they percentage it in birefringent crystals of these formations; yet they have established a coefficient of maturity of the globules according to the number of anisotrop crystals that they possess.<sup>1</sup>

In growing ovules Marza and Marza have only studied the whole cholesterol. In the mature ovule they have studied the free cholesterol, the cholesterol esters, as well as the lipoidic anisotrop esters.

**First Phase of Yolk-formation.**—The ovular membranes are poor in cholesterol. The number of anisotropic crystals of the internal theca does not go beyond twenty-eight crystals by standard unit of surface (0.10 mm<sup>2</sup>). (ovules from 50 to 2,000 microns in diameter). The juxta-epithelial layer<sup>2</sup> is slightly richer in cholesterol than the internal theca. The follicular epithelium is poorer in cholesterol than the internal theca and the juxta-epithelial layer (Table 6).

In the ooplasm only the fatty cap and the granular fat cortical layer are shown to be a little richer in cholesterol, presenting, towards the end of this period, thirty-one anisotrop crystals by standard unit of surface, and 5.7 per cent. fat-globules with

<sup>1</sup> The least rich in cholesterol are the Type 1 globules, the most rich are those of Type 4. The latter are full of anisotrop crystals.

<sup>2</sup> The juxta-epithelial layer is situated immediately above the follicular epithelium. It consists of very flat cells (Text-figs. 30, A, and 39 in Part V), which lie in layers. The number of layers varies with the diameter of the ovules.

positive reaction. The rest of the ooplasm is very poor in cholesterol.

**Second Phase of Yolk-formation.**—During this period the ovule membranes have indicated only a very slight enriching in cholesterol.

In the ooplasm only the cortical granular layer shows a certain richness in cholesterol. The primordial yolk-globules, as well as the interalveolar reticulum, are very poor in anisotrop crystals.

**Third Phase of Yolk-formation, Stage A.**—During this stage a change is seen. The membranes, to a certain extent, become enriched in cholesterol. But the changes are noticeable at the level of the yolk (Table 7 and Diagram 4).

While the primordial yolk presents only 3 per cent. globules with positive reaction, in the white yolk these globules are tripled.

Finally, the yellow yolk which appears at the end of this period is very rich in cholesterol, showing 42 per cent. positive reaction globules. Among the yellow yolk-globules of this stage Type 4 globules appear, which characterize the yolk of Stage B of this phase.

**Stage B.**—During this stage the histochemical composition of the yolk changes again.

The white cortical yolk is much richer in cholesterol than the white yolk of Stage A (Diagram 4, *wv*). On the other hand, the yellow yolk is less rich in cholesterol than during Stage A. The yolk of the parablasic region of the ovule is shown very rich in cholesterol (Diagram 4, *pb*). The latebra and the nucleus of Pander show pretty nearly the same percentage in cholesterol as the primordial yolk (Table 7 and Diagram 4). In the ovule membranes cholesterol is in considerable quantities (see Table 6).

The differences in white yolk in Stage A and B of yolk-formation show that during the evolution of the egg the yolk itself undergoes a chemical evolution before achieving the form which it presents in the yellow of the laid egg.

Yellow yolk also shows great differences in histochemical composition during these two stages.

During Stage B of the rapid-growth period the white yolk, as far as the yellow yolk, becomes enriched with Type 4 globules.<sup>1</sup>

<sup>1</sup> Figured in black, in the diagrams 4 and 5.

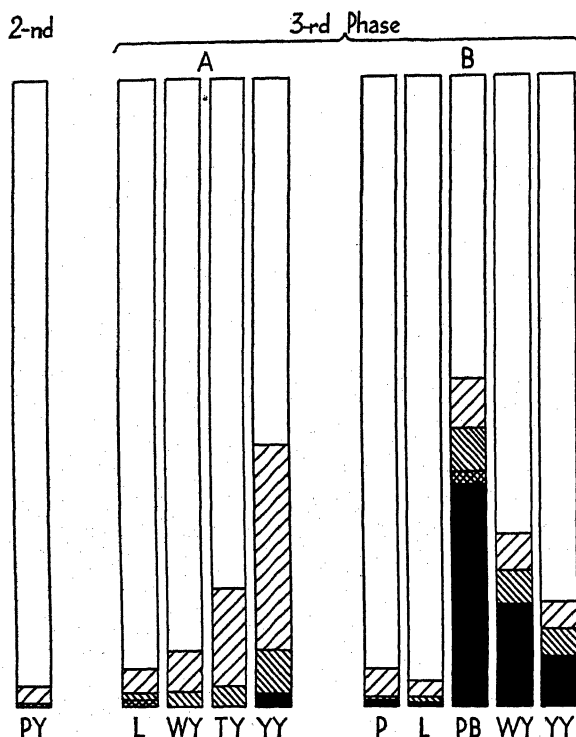


DIAGRAM 4.

Diagram showing percentage of total cholesterol in yolk-globules of the hen's egg during the second and third phases of yolk-formation. The results have been obtained through calculating the positive reaction globules in the different forms of yolk (Leulier and Noel's method for histochemical detection of cholesterol) (V. Marza and E. Marza, 'Bull. d'Histol. appl.', 1932). For the way in which the different types of positive reaction globules are represented, see inscription of following figure. PY., primordial yolk; L., latebra; WY., white yolk; TY., transitional yolk; YY., yellow yolk; P., nucleus of Pander; PB., parablastic yolk (Table 7).

Only the latebra remains at the same percentage as the primordial yolk, from which it comes, which shows us that the yolk of this part of the egg does not develop during yolk-formation.

TABLE 7.

Percentage variations in total<sup>1</sup> cholesterol of the yolk-globules, during the second and third phases of yolk-formation of the hen's egg.

<i>Period of Growth.</i>	<i>Form of Yolk.</i>	<i>Per cent. of Globules with Positive Reaction.</i>
Second Phase: . . .	Primordial yolk (PY.)	3.0
Third Phase:		
Stage A . . . . .	Latebra (L.)	5.7
	White yolk (WY.)	9.2
	Transitional yolk (TY.)	18.1
	Yellow yolk (YY.)	41.9
Stage B and eggs . . .	Latebra (L.)	4.0
	Nucleus of Pander (P.)	8.1
	Parablastic yolk (PB.)	51.5
	White cortical yolk (WY.)	28.0
	Yellow yolk (YY.)	16.4

### Yolk of the Egg.

If one were to examine the distribution of free cholesterol (CL.), cholesterol esters (EC.), and lipid anisotrop esters (EL.) in the egg after laying, one would see that the free cholesterol is found in greater quantity in the yolk of the parablastic region, and that it is in very small quantity in the latebra and in the subcircular region of the egg (nucleus of Pander) (Table 8 and Diagram 5).

The cholesterol esters are in very small quantity in the egg (Dam has put them at 12 per cent. of the total cholesterol) and they vary very little in the different forms of yolk of the laid egg (Table 8 and Diagram 5, EC.).

The ratio: free cholesterol/total cholesterol in the yellow yolk and the white yolk<sup>2</sup> is much about the same as that found by chemical process in the whole yolk.

From Table 8 it is seen that the parablastic region is almost entirely formed of free cholesterol, and that even the lipidic esters are in smaller proportion here than in the rest of the ovule. This splitting of the fatty esters is not mere chance. It proves

<sup>1</sup> Proved by Leulier and Noel's histochemical method.

<sup>2</sup> The yellow yolk constitutes the major part of the egg.

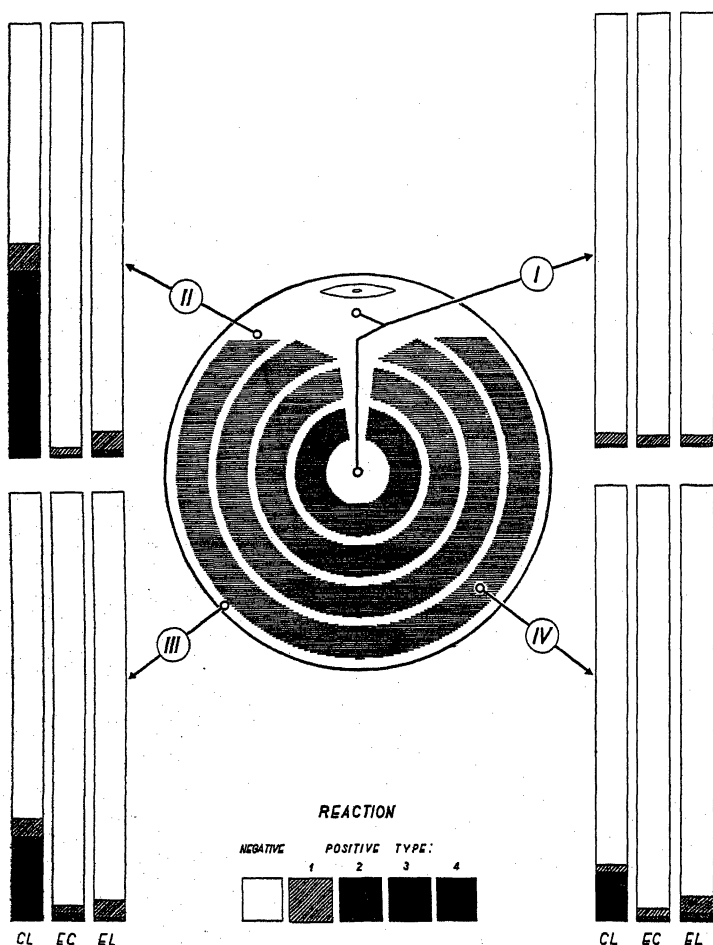


DIAGRAM 5.

Diagram of the yellow of the hen's egg, with examined regions indicated, and the percentage in each of these regions of free cholesterol (CL.), cholesterol esters (EC.), and lipoidic esters (EL.), according to Table 8. Leulier and Noel's method for histochemical detection of cholesterol (from V. Marza and E. Marza, 'Bull. d'Histol. appl.', 1932). I, latebra and under-cicatricular region (nucleus of Pander); II, parablastic region; III, white cortical yolk; IV, yellow yolk. (In the diagram published in the 'Bull. d'Histol. appl.', 1932, an error has slipped in as to the notation of white yolk and yellow yolk, an error which we have corrected.)

that the enzymes of the fertilized egg are already active at the stage when the egg is being laid.

TABLE 8.

Histochemical distribution of the free cholesterol (CL.), cholesterol esters (EC.), and lipoidic esters (EL.), in the yellow of the hen's egg.

<i>Region.</i>	CL.	EC.	EL.	<i>Free Cholesterol in per cent. of total Cholesterol.</i>
Under-cicatricular . . .	3.3	2.5	2.5	56, p. 100
Latebra . . . . .	2.6	1.3	2.0	66, p. 100
Parablastic . . . . .	49.2	2.3	5.9	95, p. 100
White yolk . . . . .	24.0	4.5	5.0	86, p. 100
Yellow yolk . . . . .	13.4	3.0	7.1	81, p. 100

### Proteins.

Little study has been made histochemically of the localization of proteins at the time of yolk-formation of the hen's egg.

Loyez states that the contents of the vacuoles that are to be found during the second phase of yolk-formation are by nature protein, and that the primordial yolk-globules are composed of lipoproteins because they are not soluble in any of the reagents used in histology (alcohol, xylol, essence of turpentine, &c.).

Konopacka confirms these affirmations. Studying the construction of the white yolk-globules the Polish author finds that the inclusions of these globules are made up of proteins and phosphatids.

Guthrie, studying yolk-formation in the eggs of several Vertebrata, arrives at the same opinion as Loyez. Guthrie gives the name of 'proteinaceous yolk-vesicles' to the clear vacuoles of the ooplasm.

The histochemical study of proteins during yolk-formation is very incomplete. It has not so far been attempted to detect either vitelline or other proteins of the hen's egg, which has determined us to undertake the task which will form the fifth part of this work.

In 1932 V. Marza, E. Marza, and L. Chiosa have proved the existence of iron and ash at the time of yolk-formation of the

hen's egg. As the iron of the yolk may form part of the vitelline molecule, we shall return to its localization when studying the proteins of the hen's egg in the fifth part of this work.

### Ash.

Histochemically, the ash has been studied by V. Marza, E. Marza, and L. Chiosa with the help of Policard's micro-incineration method.

By this method one can incinerate the histological sections, and detect from them the fixed ash (Ca, Mg, Fe, P, Si). The incinerated section is afterwards examined on a dark ground with Greenough binoculars. Except for iron ash, which is red or orange, all the others are white.

**First Phase of Yolk-formation.**—The ooplasm of eggs of 50–400 microns diameter optically appears empty on a black ground. Only the nuclear membrane and the mass of chromatin show the presence of a small quantity of greyish ash. The Balbiani body is not visible by this method; the lipoids, proteins, and other substances of which it is composed have been burnt out, leaving no trace.

In the nucleus of the frog's ovule Macallum, by his ferrocyanide method, has found iron. We have not been able to confirm the presence of iron in the nucleus of hens' ovules. Ash that we have obtained from this place is greyish, and not red or orange, as it would be if traces of iron were to be found in the chromatin.

Ash begins to appear in the form of small granulations of a light grey in the ooplasm of ovules of 400 microns diameter.

**Second Phase of Yolk-formation.**—The granulations of the cortical granular layer give light-grey ash.

In the vacuolar layer the intervacuolar layer is a little richer in ash than the cortical granular layer.

Primordial yolk-globules are rich in ash. The majority of these globules give brilliant white ash; but sometimes one sees globules whose colour is faintly yellow, indicating the existence of very small quantities of iron oxide.

**Third Phase of Yolk-formation.**—The yolk-

globules which are formed during this phase give rather few ashes in proportion to the primordial yolk-globules.

Further, the incineration of the yolk of these ovules is particularly long and laborious; it is rare to obtain whole globules—usually these burst.

Among those shrivelled up or burst, it is very difficult to distinguish the different forms of transition between the primordial yolk and the yellow yolk.

It seems to us, though, that the yolk-globules of these ovules are less rich in white ash and more rich in orange ash, which would indicate the presence of traces of iron oxide.

#### IV. CHEMISTRY OF YOLK-FORMATION.

Yolk-formation of the hen's egg has been very little studied from the chemical point of view.

No one has studied every phase of yolk-formation. The ovules of the first phase are only an insignificant mass which does not lend itself to chemical experiment. This phase of the evolution of eggs has only been examined by histochemical reactions.

The metabolism of the maturation of the hen's egg has been studied by Riddle, who has devoted several works to it. Recently Romanoff published a work on its development.

##### Water.

In the ovule membranes during growth the water has been studied by Riddle and Lawrence; in the yolk by Spohn and Riddle and by Romanoff. The membranes are very rich in water. This water varies only very little during the second and third phase of yolk-formation (Table 9, Diagram 6). On the other hand, in the yolk the water shows great variation. During the second phase it has a value equal to that of the ovular membranes (85–87 per cent.). But during Stage A of rapid growth the yolk-water falls to 60 per cent., and finishes by going down to 45 per cent. during the following stage (Table 9, Diagram 6).



These variations in water seem to be in proportion to the enriching of the yolk in fats and lipoids. The enriching of the yolk in these substances (and the impoverishment in water) is

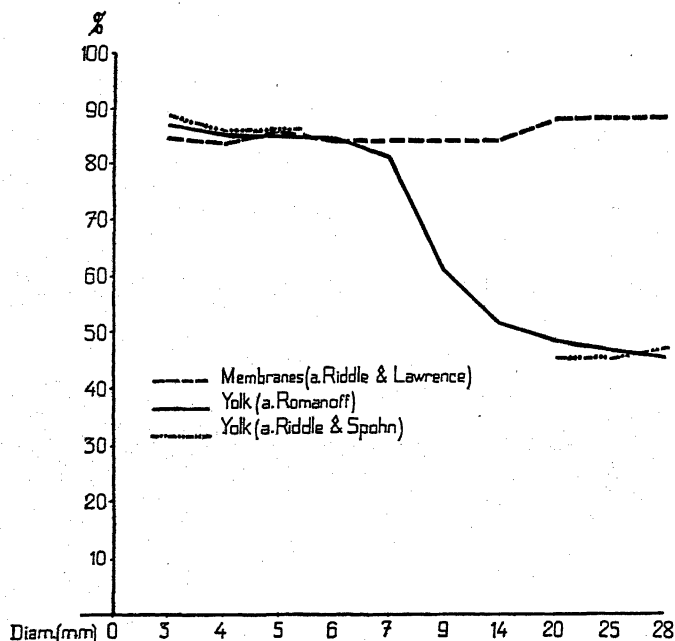


DIAGRAM 6.

The variations in water in the ovular membranes and in the yolk during the two last phases of yolk-formation of the hen's egg. (Calculated from data supplied by Riddle and Lawrence for the ovular membranes; by Spohn and Riddle, and Romanoff for the yolk.) (Table 9.)

translated morphologically by the appearance of the inclusions at the inside of the globules which are formed during Stage A of the third phase of yolk-formation.

Konopacka describes histochemically the changes that are seen in the forms of transition from primordial yolk to yellow yolk. This enrichment of the yolk in fats is interpreted by the shortening of the diameter of the inclusions, and again by the

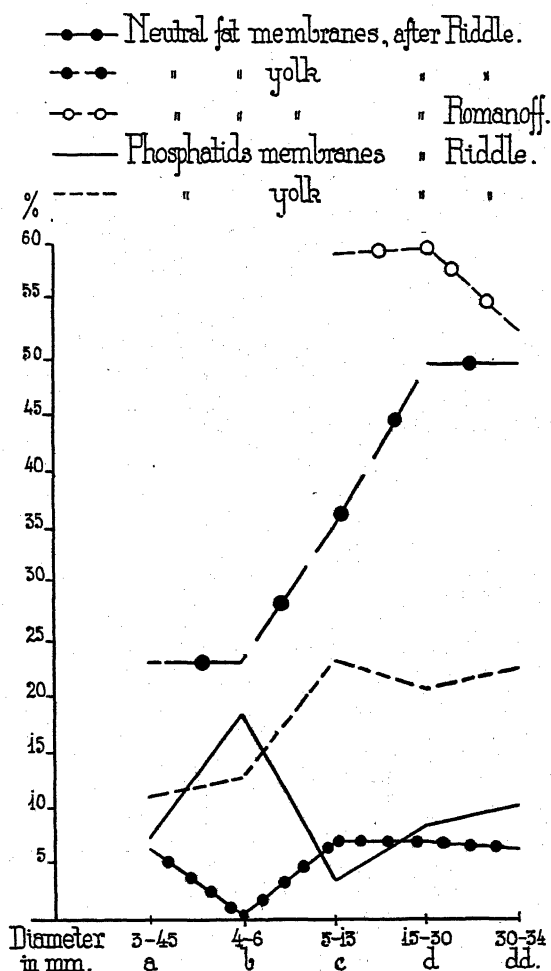


DIAGRAM 7.

The variations in lipoids and neutral fats of the ovular membranes and the yolk during the two last phases of yolk-formation. (Calculated according to data supplied by Riddle and Lawrence for the ovular membranes; by Spohn and Riddle, and Romanoff for the yolk.) (Table 10.)

invasion of the fundamental substance of vitelline globule by fat substances (Konopacka).

TABLE 9.

Percentage variations of water and dry residue in the yolk and membranes of the hen's egg during the second and third phase of yolk-formation.

<i>Diameter of Ovules (in mm.).</i>	<i>Yolk (Riddle and Spohn).</i>	<i>Water (Romanoff).</i>	<i>Ovular Membranes (Riddle and Lawrence).</i>
3	89.0	87.3 (12.7) <sup>1</sup>	85.2
4	"	85.88 (14.12)	84.05
5	"	85.65 (14.35)	85.75
6	"	84.82 (15.18)	84.18
7	"	81.43 (18.57)	"
9	"	60.7 (39.3)	"
14	45.4	51.85 (48.15)	"
20	"	48.95 (51.05)	87.93
25	"	46.5 (54.2)	"
28	"	45.46 (54.54)	"

### Dry Residue.

The residue is very reduced during the second phase of yolk-formation in the ovular membranes as well as in the yolk (Table 9, third column, numbers in parentheses).

During Stage A of the third phase of yolk-formation (ovules from 6 to 9 mm. diameter) the residue begins to increase in the yolk, and eventually at the end of this stage represents 40 per cent. of the liquid substances of the yolk. This increase shows the intensity of the changes which have taken place in the yolk from the beginning to the end of this important stage of yolk-formation.

### Phosphatids, Neutral Fats.

**Membranes.**—In ovules of 4–6 mm. diameter the lipoids and neutral fats do not undergo parallel evolution (Table 10, Diagram 7). During this time the phosphatids show a maximum, while the fat substances pass through a minimum.

This discordance in evolution is, up to the present, the only indication of chemical change in the membranes which precedes the quick growth of ovules (Riddle and Lawrence).

In larger ovules the phosphatids, as well as the neutral fats, return to their initial concentration (Table 10, Diagram 7).

<sup>1</sup> In parentheses, the dry residue numbers are represented, according to Romanoff.

**Yolk.**—During the second phase of yolk-formation the proportion of phosphatids and neutral fats does not vary. The enriching of the yolk in these substances only begins during Stage A of the third phase. But a difference can be seen between the comportment of the phosphatids and the neutral fats during the last phase of yolk-formation. The phosphatids only increase during Stage A of this phase; during Stage B their rate is almost constant. The neutral fats increase continually during these two stages of the last phase of yolk-formation.

It follows that the white yolk, the yolk of transition (Stage A), and the yellow yolk (Stage B), have almost the same richness in lipoids. What distinguishes the one from the other is their richness in neutral fats. These are in less quantity in the white yolk and yolk of transition, and in greater quantity in the yellow yolk, which entirely confirms the results obtained by Konopačka with histochemical methods.

TABLE 10.

The phosphatids and neutral fats in the ovule membranes (Riddle and Lawrence) and in the yolk (Spohn and Riddle, and Romanoff) at the time of the second and third phase of yolk-formation of the hen's egg.

<i>Diameter of Ovules (in mm.).</i>	<i>Phosphatids.</i>		<i>Neutral Fats.</i>		
	<i>Membranes.<sup>1</sup></i>	<i>Yolk.<sup>2</sup></i>	<i>Membranes.<sup>1</sup></i>	<i>Yolk.</i>	
				<i>A<sup>3</sup></i>	<i>B<sup>3</sup></i>
3-4.5	7.45	11.0	6.53	23.11	..
4-5.0	18.55	12.66	traces	„	..
5-13.0	3.65	23.11 <sup>4</sup>	7.29	35.15	59.23
15-30.0	8.55	20.58	7.05	49.51	59.92
30-35.0	10.27	22.32	6.47	„	52.4

### Proteins.

The rate of proteins in the ovular membranes only varies slightly during the second and third phase of yolk-formation (Riddle and Lawrence). The yolk of the second period is much richer in proteins than the yolk of the third phase of yolk-formation (Spohn and Riddle) (Table 11, Diagram 8, A).

<sup>1</sup> From Riddle and Lawrence.      <sup>2</sup> From Riddle and Spohn.

<sup>3</sup> From Romanoff.

<sup>4</sup> Ovules of 6.5 mm. diameter (R, Table 3, Spohn and Riddle).

If one were to compare the rate of proteins in the membranes to those in the yolk, one would see that the membranes are much richer in proteins than the yolk (Table 11).

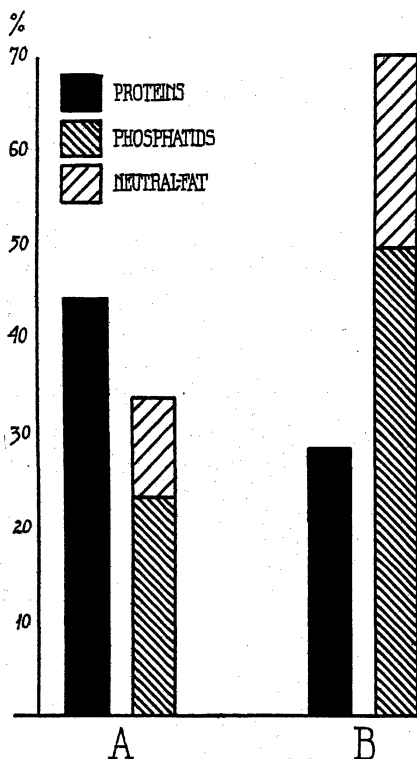


DIAGRAM 8.

Diagram of variations in proteins, neutral fats, and lipoids in the yolk, during the two last periods of yolk-formation of the hen's egg (according to data of Spohn and Riddle) (Table 12). The second phase is rich in proteins and poor in fats and lipoids, and this proportion is inverted during the third phase of yolk-formation. (A., second phase of yolk-formation; B., third phase).

There is, therefore, a concentration of proteins at the surface of the ovule; of these proteins the follicular epithelium lets a larger quantity pass into the ovule at the time of the second phase, and a smaller quantity at the time of the third phase of yolk-formation.

TABLE 11.

The proteins (in percentage of solids) in the membranes and in the yolk of the hen's egg during the second and third phase of yolk-formation.

	<i>Proteins</i> (in per cent. of solids)
Ovular membranes <sup>1</sup> . . . .	76.0 (average)
Yolk:	
Second Phase . . . .	44.4
Third Phase . . . .	28.36

If a comparative study were made of the rate of proteins and fat substances during the second and third phases of yolk-formation, it would be seen that there is an inverse ratio between the concentration of the one and that of the other during these two phases of growth.

While during the second phase the yolk is richer in proteins than in fat substances, during the third phase of yolk-formation this ratio is inverted; the fat substances are in greater quantity than the proteins (Table 12, Diagram 8).

The different forms of yolk (primordial, white, transition, and yellow) do not differ only in their morphological aspect, but also in their chemical composition.

TABLE 12.

The variations in proteins, neutral fats, and lipoids, during the second and third phase of yolk-formation of the hen's egg (Spohn and Riddle).

	<i>Yolk-formation.</i>	
	<i>Second Phase.</i>	<i>Third Phase.</i>
Proteins <sup>2</sup> . . . .	44.4	28.3
Phosphatids <sup>3</sup> . . . .	10.91	20.9
Neutral Fats . . . .	23.11	49.51

Thus it can be explained why the primordial yolk, rich in water and proteins, shows homogeneous globules, while the white yolk, the yolk of transition, and the yellow yolk show intraglobular inclusions so much finer that the form under consideration is richer in fat substances and poorer in water and proteins.

<sup>1</sup> The membranes from Riddle and Lawrence; the yolk from Spohn and Riddle.

<sup>2</sup> Proteins, neutral fats, and phosphatids in per cent. of solid substances.

<sup>3</sup> P. lecithinic.

Our opinion is that the different forms of yolk which succeed one another during the growth of the hen's egg denote as many stages in the chemical and morphological transformation of the yolk, stages which nearly approach those in ovules of other Vertebrata, and which elsewhere show (to a certain degree) the course of birds during their evolution.

### Calcium.

Romanoff has studied calcium in hen's eggs during the third phase of yolk-formation. In proportion to wet substances it is seen that calcium is in greater quantity during Stage A than during Stage B of the third phase of yolk-formation (Table 13).

### Ash.

Membrane ash varies slightly during these two last phases of yolk-formation (Riddle and Lawrence).

Ash is in less quantity in the primordial yolk than in the yolk which is formed during the third phase of yolk-formation (Riddle and Spohn) (Table 14).

### pH.

pH has been studied by Romanoff during the third phase of yolk-formation. During Stage A of this phase the yolk has a more alkaline reaction than during Stage B (Table 13).

TABLE 13.

Calcium (in percentage of wet substances) and pH during the third phase of ovule formation of the hen's egg (Romanoff).

<i>Third Phase of Yolk-formation.</i>	<i>Diameter of Ovules (in mm.).</i>	Ca.	pH.
Stage A	9.0	4.77	6.289
Stage B	14.75	3.98	6.137
	20.25	2.4	6.027
	25.0	1.93	5.917
	28.0	2.59	5.9

TABLE 14.

Ash of the membranes and yolk of the hen's egg during the second and third phase of yolk-formation.

<i>Diameter of Ovules (in mm.).</i>	<i>Membranes (Riddle and Lawrence).</i>	<i>Yolk (Spohn and Riddle).</i>
Second Phase:		
3-4.5 . . . .	6.05	6.03
4-6.0 . . . .	3.78	"
Third Phase:		
5-13.0 . . . .	2.29	
15-30.0 . . . .	2.72	10.58
30-35.0 . . . .	3.70	"





# The Formation of the Hen's Egg.

## V. Histochemistry of Yolk-Formation. Proteins.<sup>1</sup>

By  
V. D. Marza

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With Text-figures 21-48.

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<sup>1</sup> The first four parts of this paper appeared in the previous number of this Journal: vol. 78, p. 133.

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### INTRODUCTION.

THE best known protein in the yolk of the hen's egg is vitellin. Next to that one finds livetin, Gross's protein,<sup>1</sup> and the nucleoproteins. According to Hugounencq and Morel there is 1.40 gr. of vitellin in the hen's egg. Livetin is in lesser quantity. The quantity of nucleoproteins does not go beyond 2 mgr.

Vitellin contains iron and phosphorus. Livetin does not contain iron; this protein is poorer in phosphorus than vitellin.

We will not dwell here on the discovery and constitution of vitellin and livetin. These two questions are dealt with very competently in J. Needham's 'Chemical Embryology' (1931).

What are of interest to us, from a histochemical point of view, are the reactions and solubility of these two proteins, vitellin and livetin. Due to various reactions and solubilities, we have been able to detect separately, and also determine the localization of, the variations in these proteins in the different forms of yolk.

## VITELLIN AND LIVETIN

### REACTIONS AND SOLUBILITY.

Here are the properties of these two proteins in a few words:

(1) For a long time Hoppe-Seyler and Miescher have affirmed that vitellin behaves like a globulin; it is insoluble in water and soluble in dilute solutions of neutral salts. Vitellin gives the biuret reaction.

(2) According to Kay and Marshall, livetin is a pseudo-globulin, soluble in water as well as in NaCl solutions.

(3) In the yolk of the hen's egg the proteins are joined to phosphatids. For a long time it was believed that this connexion was of a chemical order. The researches of Fischer and Hooker

<sup>1</sup> Latterly, Kay has found that Gross's protein is identical with livetin. One finds, therefore, only three proteins in the hen's egg—vitellin, livetin, and nucleoproteins. The first is a phospho-protein.

(1926) have ended this idea, and have shown (a) that the linkage between the vitellin and phosphatids is of a physical order; (b) that alcohol destroys this linkage.

#### HISTOCHEMICAL LOCALIZATION. HISTORICAL ACCOUNT.

The number of authors who have tried to localize vitellin histochemically is very small.

In the two last decades of the past century, in a discussion on the existence of a nucleus in the white yolk-globules, Miescher stated that the granulations that are seen in this form of yolk are constituted by vitellin (or nucleovitellin, according to the nomenclature of Miescher).

The cellular conception of the vitellin globule has completely fallen into disuse. But the observations of a histochemical order made by Miescher, on the occasion of this discussion, deserve recall here. According to Miescher:

(a) The small slabs of yolk of the egg of *Pristiurus* possess a cortical region, consisting of a protein insoluble in NaCl, and a central region of which the protein is soluble in NaCl (like vitellin). According to Miescher, vitellin is only found at the centre of the small slab, the periphery being made up of another protein.

(b) Vitellin would be localized in the spherules that one finds inside the white yolk-globules of the hen's egg. But as white yolk is in small quantity in the hen's egg, Miescher deduces from that, that the yellow yolk-globules must also contain vitellin. By analogy with the localization of vitellin in white yolk-globules, he deduces that the vitellin must be localized in the fine granulations which fill the yellow yolk-globules.

(c) In the eggs of the salmon, not only the yolk globules, but even the interglobular liquid must contain vitellin, because it precipitates in water, and it is insoluble in solution at 0.75 per cent. NaCl.

In a histological study Steudel and Osata have put forth the idea that several forms of vitellin might exist in the same egg.

We do not know by what reactions and methods these authors have succeeded in establishing their assertion, their work not being accessible to us. As we shall see later, there is still no

known method for the direct histochemical detection of vitellin, and still less for testing the different varieties of vitellin, if these varieties really exist.

If we have only quoted Miescher, Steudel, and Osata, it is that the other authors engaged on vitellin have only made suppositions as to its histochemical localization.

### PROTEINS HISTOCHEMICALLY REVEALED AND METHODS USED

(1) *Vitellin and Livetin*.—We have no methods for demonstrating vitellin directly. But we think we have succeeded in revealing histochemically this substance, basing our conviction on two of its properties:

(a) Vitellin reacts like a globulin.

(b) Vitellin contains iron. This metal is only found in vitellin. No other proteins of the yolk of the hen's egg possess it.

Globulin reaction is also given by livetin, which, as we have mentioned earlier, shows pseudoglobulin reaction. We can, however, distinguish vitellin from livetin, thanks to the fact that livetin is soluble in water, while vitellin is not. We have used this differential solubility to give an account of the rate and localization of globulins (of the vitellin kind) and of pseudoglobulins (of the livetin kind) in the yolk of the hen's egg.

For testing globulins, pseudoglobulins, and cytosols, we have used Unna's method with methyl-green pyronine. The pyronine gives coloured reaction, not only with globulins and pseudoglobulins, but also with cytosols (P. Unna), simpler protein molecules.

*Vitellin Iron*.—This compound was detected by Macallum's methods and by Policard's micro-incineration method. The methods of Macallum test for organic and inorganic iron, either under the form of Prussian blue, or under the form of Turnbull's blue, iron sulphide, iron rhodanate, or ferric lac of haemotoxylin.

Policard's micro-incineration method shows the iron as oxide red among the white ash of the incinerated section.

The iron only being localized in the hematogen, its presence allows us to affirm, with certainty, the appearance and localization of this part of the vitellin.

(2) Nucleoproteins.—The nucleoproteins have been proved by Unna's method with methyl-green pyronine, and by Feulgen's method, for the histochemical detection of the thymonucleic acid.

Pyronine reacts with globulins, pseudoglobulins, and cytosols. Methyl-green reacts with the nucleoproteins according to Unna.

(3) Detection of Proteins with Basic Reaction.—Unna's Bordeaux B method has allowed us to detect another series of proteins with basic reaction.

#### FIXATIVE.

At the time of these researches we have used, for preference, hens' ovaries fixed in alcohol and, to a less degree, ovaries fixed in acetic sublimate.

Alcohol is a very bad histological fixer, but in histochemistry it is used very often in the study of glycogen, proteins, and certain metals or metalloids (Fe, K, Ca, S, &c.). Apart from that, alcohol has the reputation of separating lipoids from vitellin in the yolk of the hen's egg (Fischer and Hooker).

Alcohol contracts the tissues, and in small ovules (whose ovoplasm is very fluid) alcoholic fixation provokes the condensation of the ovoplasm to the pole opposite the germinative epithelium of the ovary (Text-fig. 24, *d*). This condensation, which is only an artifact, is only visible in coloured preparations, where it is conspicuous owing to a great concentration of colour at its surface.

Although the acetic sublimate is an excellent fixative, we have only used it on rare occasions, because of its action on the fats, and also because on a subject fixed by alcohol, one can execute many more histochemical reactions than on a subject fixed by sublimate.

#### MATERIAL USED.

We have used ovaries of adult hens; 20 ovaries have been fixed in 96% alcohol and 5 ovaries in acetic sublimate.

On sections (in series) of these ovaries we have carried out the reaction of acid proteins (globulins, pseudoglobulins, cytosols), reaction of basic proteins, nuclear reaction, and iron reactions.

A. DETECTION OF PROTEINS WITH ACID REACTION.  
(Vitellin, Livetin, Globulins, Pseudoglobulins,  
and Cytoses.) Method.

For the detection of acid proteins, Unna has recommended the method of methyl-green pyronine.

Pyronine acts in the same way on globulins and cytoscs, colouring them red. These same proteins can both be found in the same tissue. To be able to account for the richness of an organ in each of these proteins, one colours a first section with pyronine; a second section is coloured only after having dissolved one of these proteins. These two sections are afterwards compared. The difference in colour indicates richness of the organ in the dissolved protein. The colour presented by an organ after the dissolution of one of these proteins indicates the richness of this organ in the insoluble protein.

The process of selective dissolution of a substance has been called chromolysis by Unna. Chromolysis well carried out can do very useful service in histochemistry.

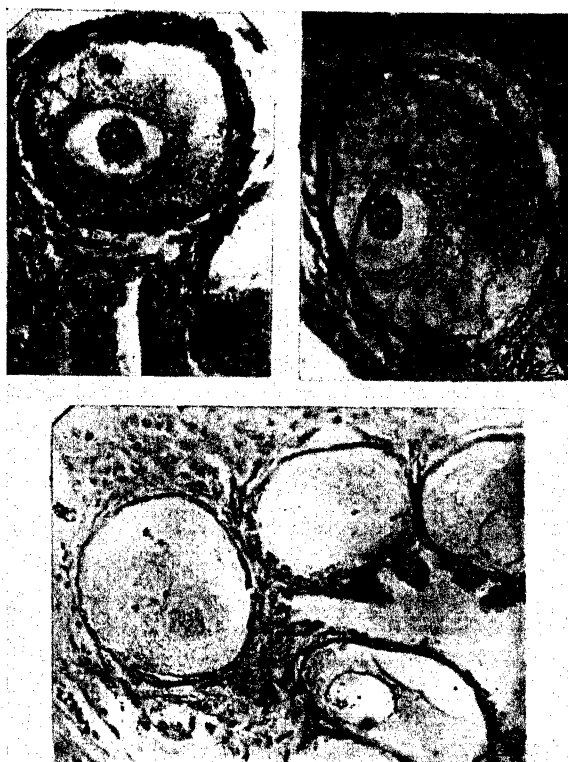
We have used three series of sections.

(a) A first series of sections after removal of paraffin is coloured with pyronine before every chromolysis. We designate these sections by the name of 'total acid proteins' or more simply 'T.A.P.'

(b) On a second series of sections the cytoscs and pseudoglobulins are dissolved. These two proteins are soluble in distilled water (first chromolysis). The technique employed is as follows: the section, after removal of paraffin, is plunged into a glass of sterilized water, which is carried to the incubator at 40° C., where the section remains (in the water) for 24 hours.<sup>1</sup> Afterwards, it is coloured with methyl-green pyronine. Under the action of distilled water, only the globulins and vitellin remain, as these are not soluble in sterilized water.

Afterwards, after the first chromolysis, the section is compared with those of Group T.A.P. Those elements which have pos-

<sup>1</sup> It is as well to work with a sterilized medium (glass and solutions of distilled water and sodium chloride). Thus the development of fungoid growth or certain microbes, which can modify the colorability of the section, is avoided.



TEXT-FIGS. 21-3. Acid Proteins in Small Ovules.

Fig. 21.—Ovary No. 75. Fixative—96% alcohol. Total acid proteins (Unna's method with pyronine methyl-green). Ovule of  $79 \times 84$  microns diameter ( $6 \times D$ ). Nuclei of internal theca, follicular epithelium, and nucleolus of ovule nucleus show more intense reactions; ovoplasm, nuclear mass (ovule nucleus), cytoplasm of internal theca cells show much less intense reaction.

Fig. 22.—Ovary No. 24. Fixative—96% alcohol. Acid proteins after first chromolysis (Unna's method with pyronine methyl-green). Ovule of  $85 \times 104$  microns diameter ( $6 \times D$ ). Colours of membrane nuclei and cytoplasm of follicular epithelium have decreased. Ovoplasm and chromatic mass of ovule nucleus have remained unchanged.

Fig. 23.—Ovary No. 24. Fixative—96% alcohol. Acid proteins after second chromolysis (Unna's method with pyronine methyl-green). Group of ovules of approximately 100 microns diameter ( $5 \times D$ ). After second chromolysis, cytoplasm of membrane cells, ovoplasm, ovule nucleus no longer take pyronine.



sedged cytosols or pseudoglobulins take pyronine less well (cf. Text-fig. 22 with fig. 21).

(c) Unna recommends a second chromolysis as well, which is, as a matter of fact, only an operation of control. This second chromolysis is carried out as follows: the paraffin is removed from a third series of sections, and put in a glass filled with a solution of 2 per cent. NaCl (sterilized). The glass and the sections are put in the incubator at 40° C. for 24 hours.

Under these conditions the globulins, as well as the cytosols, are dissolved, and pass into the solution of NaCl. Only the nucleoproteins remain in the section. A section which has remained in the solution of NaCl ought not to stain with pyronine. Only methyl-green ought to colour the nuclei blue-green (Text-figs. 23, 26, and 27).

Sometimes, after the second chromolysis, one sees in certain elements (condensation of the ovoplasm) a violet colour. This colour is not due to pyronine, but to methyl-violet, which is frequently present as an impurity. The violet colour, if it is present, makes the interpretation of results difficult. It is necessary to eliminate the methyl-violet.

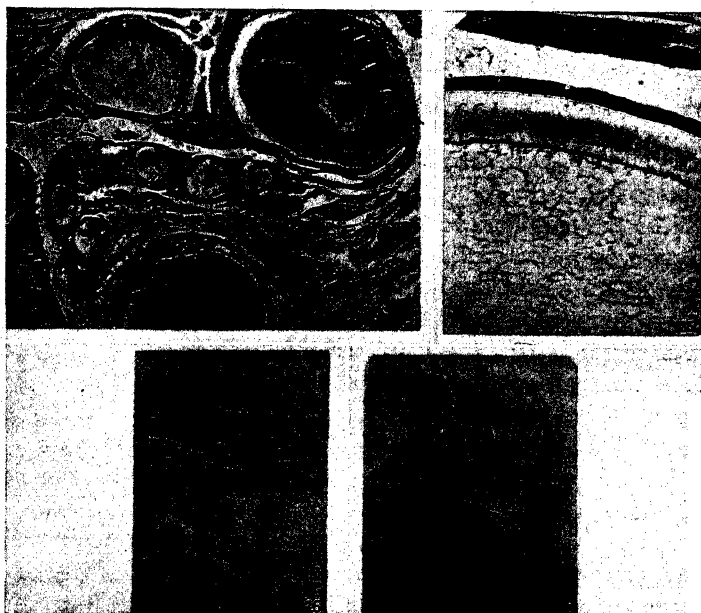
Unna recommends the purification of methyl-green by shaking it up with chloroform. It is left to sediment, and afterwards the violet liquid (containing the methyl violet) is decanted. It is necessary to repeat this extraction eight to ten times, until the chloroform is no longer coloured violet.<sup>1</sup>

### Standardization of the Method.

Unna's method with methyl-green pyronine gives varying colours in action with different elements, of which certain are of a technical order, and certain others histochemical. These colours vary: (1) with the time of contact of the subject with the stain; (2) with the speed of dehydration by alcohol of the coloured section; (3) with the thickness of the section; (4) with

<sup>1</sup> Unna gives for his stain the following formula:

Methyl-green (purified)	. . . . .	0.15 gr.
Pyronine	. . . . .	0.25 gr.
96% Alcohol	. . . . .	2.5 c.c.
Carbolic acid (0.5 per cent.)	. . . . .	100.0 c.c.



TEXT-FIGS. 24-7. Acid Proteins in Larger Ovules.

Fig. 24.—Ovary No. 75. Fixative—96% alcohol. Total acid proteins (Unna's method with pyronine methyl-green) ( $6\times A$ ). Aspect of a portion of ovary. In smaller ovules, Balbiani body is seen (1, 2). In larger ovules densification (*d*), in form of a concentration of colour at pole of nucleus opposite germinal epithelium (*eg.*).

Fig. 25.—Ovary No. 45. Fixative—96% alcohol. Total acid proteins (Unna's method). Ovule of  $2.5\times 2.8$  mm. diameter (oc. comp.  $8\times A$ ). Between granular cortical layer (GCL) and central vacuolar layer (VR.) there is a concentration of proteins. Internal theca, juxta-epithelial layer, and follicular epithelium show a much more intense reaction than two layers of ovoplasm (ovule during Stage 'A' of the second phase of yolk-formation).

Figs. 26 and 27.—Ovary No. 45. Fixative—96% alcohol. Acid proteins after second chromolysis (Unna's method) ( $5\times D$ ). Only nuclei of internal theca, juxta-epithelial layer, and follicular epithelium are coloured by methyl-green. Cytoplasm of these cells as well as yolk-globules and ovoplasm have lost, under dissolving action of NaCl, proteins colourable by pyronine.

the affinity of the tissues for pyronine, that is to say, with the richness of the organs in globulins and cytosols.

No. 3 is avoided by always cutting the sections of the same thickness (we section the organs, paraffin included, at 8 microns).

To avoid the mistakes due to Nos. 1 and 2 we have tried to standardize the method. With this aim we have used two procedures.

The first procedure consists of using a system of three metallic clamps, by means of which one can manipulate several slides at a time. This contrivance has enabled us to colour and dehydrate at the same time six sections, one clamp holding two slides, before each chromolysis (T.A.P.); a second clamp holding two slides which have undergone the action of distilled water (first chromolysis); finally, the third clamp holding two slides which have undergone the action of the solution of NaCl (second chromolysis).

The slides are marked with a diamond to eliminate the risk of mixing them.

Thanks to this contrivance, these six slides are plunged at the same time into the glass containing the stain (methyl-green pyronine), where they remain for 20 minutes, after which they are rapidly washed in water for 1-2 seconds; the sections are later passed through four glasses of 96% alcohol (5 seconds in each) which is sufficient for complete dehydration; after which the sections are passed through xylol, which has no further dissolving power on the pyronine. At this stage, the slides are taken from their clamps, rinsed two or three times in xylol, and the sections mounted in Canada balsam.

In this way the differences in colour, due to varying conditions of coloration and dehydration (Nos. 1 and 2) of the sections to be compared, are completely avoided.

To avoid variations of a technical order, it rests with us to examine the richness of the tissues in globulins and cytosols by their affinity for pyronine, which constitutes the aim of this work.

The second procedure is the technique recommended by Unna himself. Following this method, each section is coloured and dehydrated separately.

This last procedure is less costly than the preceding, but the results are much less precise. One must be accustomed to the

variations in colour in the ovules, to be able to use the results obtained by the second procedure profitably. Its only advantage is that it does not require a great waste of reagents (stain, alcohol). On the other hand, in using the second procedure, one almost always has a number of doubtful results.

### Scale of Colouring.

In order to study the variations in colour in the different regions of the ovules, we have used a colour scale conventionally established. We have marked by figures seven tints: the palest by number 0.5, the strongest by number 3.5, and in between the other tints by numbers: 1, 1.5, 2, 2.5, and 3. This scale is represented in the co-ordinates of all our diagrams.

For pyronine we have established four staining intensities:

- (1) Pale pink (0.5), weakly positive reaction.
- (2) Light red (1.0), positive reaction.
- (3) Red (2.0), intense reaction.
- (4) Deep red (3), very intense reaction.

Certain elements of the hen's ovary show a constant colour. We have used these colours as indices of comparison with elements of variable histochemical structure.

In the T.A.P. slides we have noted constant colour (for pyronine) in the following elements:

Cytoplasm of the ovular stroma 0.5.

Vitelline Balbiani body 1.0.

Nucleolus (nuclei of the follicular cells) 2, 5-3, 0.

Cytoplasm of the small interstitial cells 3.

Among these colours, those shown by the other tissues or regions examined are graded.

For methyl-green, which never gives such intense colouring, we give three grades in colour: 0.5, 1, 1.5. The intensity of these colours corresponds to the analogous colours given by pyronine.

### Regions Examined.

We have examined the ovule membranes and the ovoplasm at the same time.

The ovule membranes are:

- (1) The follicular theca, or internal theca (IT.) (Pearland Boring).

- (2) The juxta-epithelial layer, made up of very flat thecal cells, situated immediately above the follicular epithelium of the ovule (JEL.).
- (3) Follicular epithelium (FE.).
- (4) Zona radiata, when it is visible. This formation only appears in larger ovules (end of first phase of yolk-formation, second phase of yolk-formation). The radiated zone is no longer seen round ovules of accelerated growth (third phase of yolk-formation).

Ovoplasm.—(a) In ovules of 30 microns to 2 mm. diameter, we have examined: (1) the ovoplasm properly called (o.), (2) the vitelline Balbiani body (BB.), in ovules of 50–150 microns diameter, and (3) the ovule nucleus (ON.) with its membrane (M.), its chromatin (CH.), and its nucleolus (NC.).

(b) In ovules 2–6 mm. diameter (second phase of yolk-formation) we have examined: (1) the granular cortical layer (GCL.), (2) the central vacuolar layer (VL.), and (3) the yolk-globules of these vacuoles, our primordial yolk (PY.).

(c) In ovules from 7 to 20 mm. diameter (third phase of yolk-formation) we have examined: (1) the white cortical yolk (WY.), (2) transition yolk, (3) yellow yolk (YY.), and (4) latebra, with its layers (L.) as well as the nucleus of Pander (or under-citricular yolk) (PN.).

In the rest of the ovary we have examined: (1) the ovarian stroma, and (2) the interstitial cells.

These last will form the subject of a separate study.

### Results.

#### (a) Nuclei of Ovular Membranes.

The colours of the internal theca nuclei, of the juxta-epithelial layer and the follicular epithelium, vary only slightly during the ovule's growth. From this point of view the nuclei contrast with the cytoplasm in the same cells, which shows intense variations during yolk-formation.

The colours of the nuclei of ovular membranes are described in Table 15. In this table we show the manner adopted by us for calculating the richness in globulins and cytosols of the elements examined (Table 16).

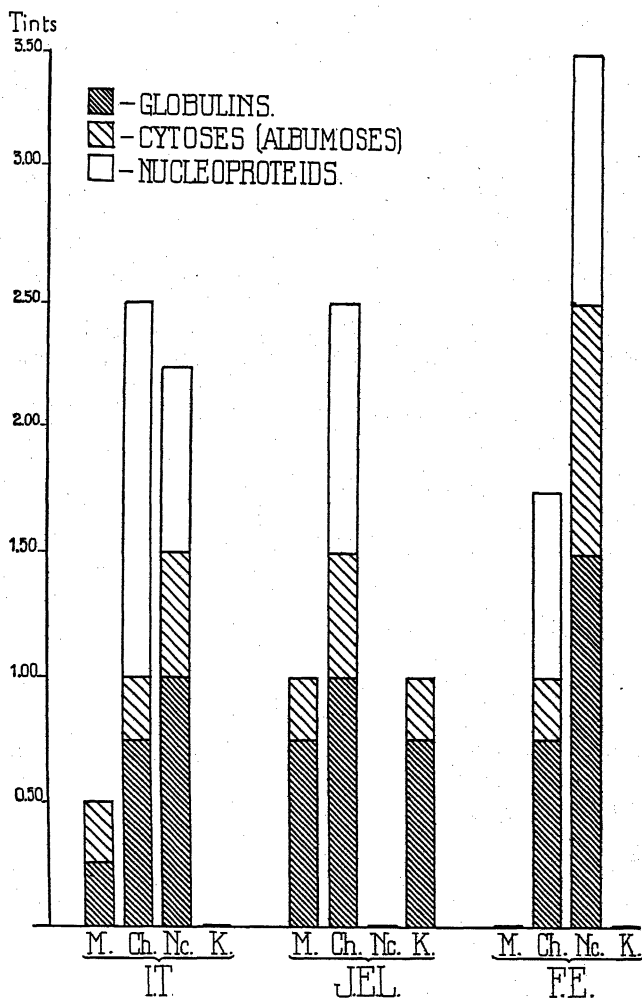


DIAGRAM 9.

The average colours of acid proteins (globulins, cytos or albumoses, and nucleoproteins) in the nuclei of ovular membranes (Tables 15 and 16). Co-ordinate: scale of colouring M., nuclear membrane; CH., chromatin; NC., nucleolus; K., karyoplasm; IT., internal theca; JEL., juxta-epithelial layer; FE., follicular epithelium. There are great differences in histochemical composition existing between the four nuclear elements of these three ovular membranes.

TABLE 15.

Acid-reacting proteins in nuclei of ovular membranes during the ovule's growth. A = total acid proteins (globulins, pseudoglobulins, cytosols, and nucleoproteins). I = acid proteins after first chromolysis (globulins and nucleoproteins). II = acid proteins after the second chromolysis (nucleoproteins).

	<i>Internal Theca.</i>			<i>Juxta-epithelial Layer.</i>			<i>Follicular Epithelium.</i>		
	A	I	II	A	I	II	A	I	II
Pyronine.									
Membrane . .	0.5	0.25	0	1.0	0.75	0	0	0	0
Chromatin . .	1.0	0.75	0	1.5	1.0	0	1.0	0.75	0
Nucleolus . .	1.5	1.0	0	?	?	?	2.5	1.0	0
Karyoplasm . .	0	0	0	1.0	0.75	0	0	0	0
Methyl-green.									
Membrane . .	..	..	0	..	..	0	..	..	0
Chromatin . .	..	..	1.5	..	..	1.0	..	..	0.75
Nucleolus . .	..	..	0.75	..	..	0	..	..	1.0
Karyoplasm . .	..	..	0	..	..	0	..	..	0

In this table, column 1 (pyronine) represents the colour that the globulins should have. The difference between Column A and Column I (pyronine) shows the colour due to cytosols and pseudoglobulins. Column II (methyl-green) shows the colour of the nucleoproteins.

It is necessary to state that in this and following tables the colour of each of these elements represents the average colour obtained from examination of 536 ovules: of which 206 for total acid proteins (T.A.P.); 225, for ovules which have undergone first chromolysis; and 105, for ovules having undergone second chromolysis.

From Table 16 and Diagram 9 it is seen that there are great differences between the histochemical construction of the nuclei of ovular membranes. The nuclei of the juxta-epithelial layer differ in histochemical construction from those of the internal theca. Morphologically, the differences between the cells of these two layers are less clear, the juxta-epithelial layer being made up of flatter cells than those of the internal theca.

It is also seen that in nuclei of the follicular cells the membrane and karyoplasm do not possess acid proteins. On the other hand, the chromatin, and above all the nucleolus, are particularly rich in these proteins (see Diagram 9).

TABLE 16.

Average colours of acid proteins (nuclei of ovular membranes) deduced from Columns A, I, and II of Table 15.

	<i>Globulins.</i>	<i>Cytoses.</i>	<i>Nucleo-proteins.</i>
<b>Internal Theca.</b>			
Membrane . . . .	0.25	0.25	0
Chromatin. . . .	0.75	0.25	1.5
Nucleolus . . . .	1.0	0.5	0.75
Karyoplasm . . . .	0	0	0
<b>Juxta-epithelial Layer.</b>			
Membrane . . . .	0.75	0.25	0
Chromatin. . . .	1.0	0.5	1.0
Nucleolus . . . .	0	0	0
Karyoplasm . . . .	0.75	0.25	0
<b>Follicular Epithelium.</b>			
Membrane . . . .	0	0	0
Chromatin. . . .	0.75	0.25	0.75
Nucleolus . . . .	1.50	1.0	1.0
Karyoplasm . . . .	0	0	0

(b) Cytoplasm of Membranes, Ovoplasm, and Yolk.

The ovarian stroma is made up of connective tissue cells, of which the cytoplasm is rather poor in acid proteins. The average colours of the cytoplasm of the stroma cells are: A = 0.5, I = 0.25, II = 0; which signifies that the globulins and cytosés are in very small quantity on the level of the stroma.

The colours of the stroma contrast with the colours of the ovular membranes, which are much more intense. In the case of the latter this is occasioned probably by an enriching of the membranes in acid proteins, under the influence of the proteins which diffuse from blood to the ovule. Thus the blood capillaries are more numerous in ovular membranes (internal theca, juxta-epithelial layer) than in the stroma, even in the case of small ovules which have very reduced vascularization.

First Phase of Yolk-formation.

Ovules from 25 to 50 microns Diameter.—Globulins and cytosés are in very small quantity on the level



of the internal theca cytoplasm. The juxta-epithelial layer has not yet made its appearance.

The follicular epithelium, with very flat cells, shows a certain wealth in globulins (Table 17).

The ovoplasm does not show the presence of acid proteins.

In the nucleus of the ovule, the nucleolus shows an intensely positive reaction, due mainly to the globulins.

The Balbiani body is not visible.

Ovules from 50 to 100 microns Diameter.—As regards the internal theca, the juxta-epithelial layer, and the follicular epithelium, no differences are discernible between these ovules and ovules of 25–50 microns diameter (Table 17 and Diagrams 10, 11, and 12).

The ovoplasm shows negative reaction.

The Balbiani body is made up of globulins; cytosoles do not enter into its composition (Table 18 and Diagram 14, p. 215).

The ovule nucleus has not changed colour (Text-fig. 21).

Ovules from 100 to 300 microns Diameter.—The internal theca has not altered its constitution histochemically; it has kept the same composition as in smaller ovules (Table 17 and Diagram 10).

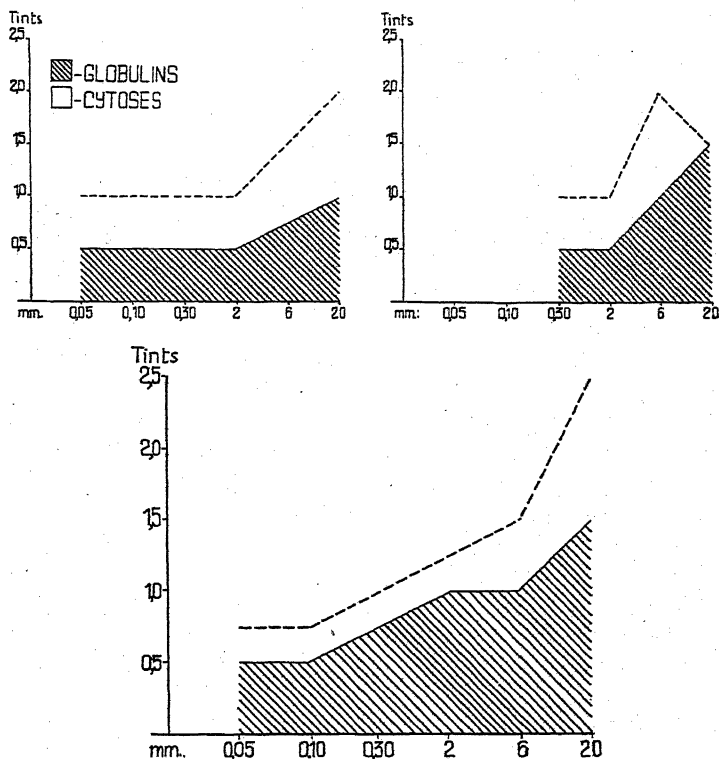
The juxta-epithelial layer makes its appearance in the form of a membrane made up of one or two layers of very flat cells (Text-fig. 30, *a*), in whose cytoplasm the cytosoles and globulins are found in equal quantity (Diagram 11).

The quantity of globulins in the follicular epithelium has increased to the point of surpassing the cytosoles (Diagram 12).

The colour of the ovoplasm is weakly positive. It is hardly coloured pink, and the colour only disappears after the second chromolysis; this means that the ovoplasm is beginning to possess globulins (Diagram 14, *o*).

The ovoplasm globulins can have two origins: (1) coming from the disintegration of the Balbiani body, whose globulins are dispersed into the ovoplasm; (2) another lot of ovoplasmic globulins of exogenous origin, derived from the proteins diffused from the blood. Judging by the size of the ovule in proportion to the Balbiani body, it seems to us that globulins of exogenous origin predominate in the ovoplasm.

The colours of the ovule nucleus are the same as in former stages, except as regards the nucleolus, which are only rarely seen in ovules which have reached this stage of evolution.



DIAGRAMS 10, 11, AND 12.

The average colours of acid proteins in the cytoplasm of the ovular membranes during yolk-formation of the hen's egg (Table 17, p. 208). (Unna's method for histochemical detection of acid proteins.)

Co-ordinates: scale of colouring. Abcissae: diameter of the ovules in mm.

Diagram 10 shows the richness in globulins and cytosols of the internal theca; Diagram 11 of the juxta-epithelial layer; and Diagram 12 of the follicular epithelium.

Ovules from 300 to 2,000 microns Diameter.— Neither the internal theca nor the juxta-epithelial layer have changed composition in these ovules. On the other hand, in the

cytoplasm of the follicular epithelium an enriching in globulins is to be noted, while the cytoseres are found in the same quantity as in smaller ovules (Diagram 12, Table 17).

In proportion to the vascularization of the egg, which begins now to be more important (Text-fig. 45), this enriching in proteins in the follicular epithelium is somewhat reduced. This phenomenon probably means that the blood capillaries or follicular epithelium are not very permeable to proteins; on the other hand, these membranes appear to be very permeable to fat substances, which are found in great quantity in the ovoplasm of eggs having this diameter (see first part of this work, paragraph concerning histochemistry of yolk-formation).

The ovoplasm shows a clear pink colour, exclusive of globulins (Diagram 14, o., and Table 18), but in comparison with ovular membranes the ovoplasm possesses only a smallish quantity of protein.

The nucleus of the ovule shows histochemical and morphological changes. The chromatin has lost its clarity, being discernible only with difficulty; nor is the nucleolus any longer seen in this method (see also chapter devoted to nuclear changes in the first part of this work).

TABLE 17.

The average colours of acid proteins in the cytoplasm of the ovular membranes during the yolk-formation of the hen's egg. GL., globulins; CY., cytoseres.

<i>Diameter of Ovules (in mm.).</i>	<i>Internal Theca.</i>		<i>Juxta-epithe- lial Layer.</i>		<i>Follicular Epithelium.</i>		<i>Radiated Zone.</i>	
	GL.	CY.	GL.	CY.	GL.	CY.	GL.	CY.
0.050 . . .	0.5	0.5	..	..	0.5	0.25	..	..
0.100 . . .	0.5	0.5	..	..	0.5	0.25	..	..
0.300 . . .	0.5	0.5	0.5	0.5	0.75	0.25	..	..
2.0 . . .	0.5	0.5	0.5	0.5	1.0	0.25	0.25	0.25
6.0 . . .	0.75	0.75	1.0	1.0	1.0	0.5	..	..
20.0 . . .	1.0	1.0	1.5	0	1.5	1.0	..	..

### Second Phase of Yolk-formation.

Ovules from 2 to 6 mm. Diameter.—The internal theca of these ovules, as well as the juxta-epithelial layer, show deeper colouring than in the preceding stage, but the proportion

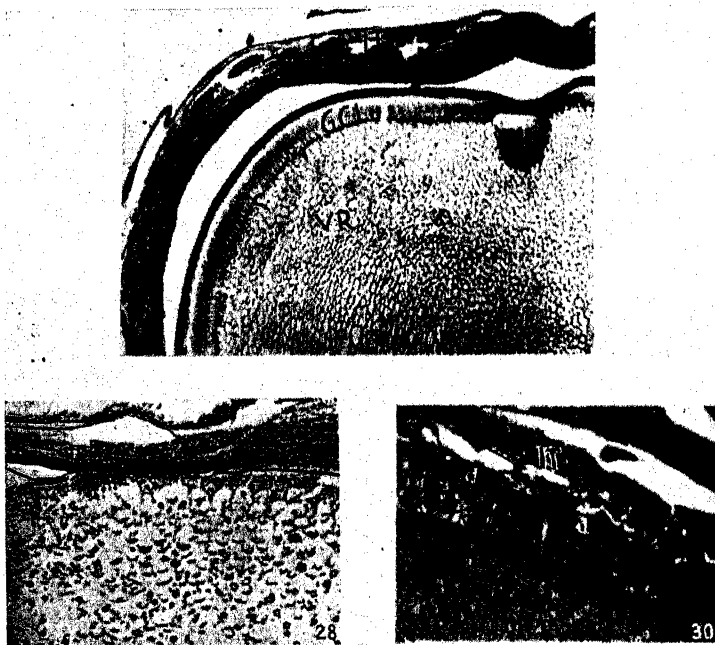
TABLE 18

The average colours of acid proteins in the ovoplasm and the yolk during the three phases of yolk-formation

	<i>Globulins.</i>	<i>Cytoses.</i>	<i>Nucleo-proteins.</i>
<b>First Phase of Yolk-formation.</b>			
Ovoplasm . . . . .	0.25	0	0
Balbani body . . . . .	1.0	0	0
Densification . . . . .	1.5	0	0
<b>Ovule Nucleus.</b>			
Membrane . . . . .	0.75	0.25	0
Chromatin . . . . .	0.25	0.25	0.5
Nucleolus . . . . .	1.5	0.5	..
<b>Second Phase of Yolk-formation.</b>			
Granular cortical layer . . . . .	0.25	0.25	0
Vacuolar reticulum . . . . .	0.25	0.25	0
<b>Primordial Yolk.</b>			
Centre of globule . . . . .	1.0	0	0
Periphery . . . . .	0	0	0.25
<b>Third Phase of Yolk-formation.</b>			
<b>White Yolk.</b>			
Fundamental substance . . . . .	0.25	0.25	0
Inclusions . . . . .	0.25	0.25	0.25
<b>Yellow Yolk</b>			
Transitional Yolk.	0.5	0.25	0
<b>Vacuoles</b>			
Vacuoles . . . . .	0	0	0
Fundamental substance . . . . .	0.5	0.25	0
<b>Latebra (Layer D).</b>			
Fundamental substance . . . . .	0.25	0.25	0
Inclusions . . . . .	0.25	0.25	0.25
<b>(Layer B).</b>			
Centre of globule . . . . .	1.0	0	0
Periphery . . . . .	0	0	0.25

of globulins and cytoseres remains the same as in less developed ovules (Table 17 and Diagram 10).

Sometimes it is seen that the colour shown by the juxta-epithelial layer is more intense than that of the internal theca (Text-figs. 28 and 29), but the differences are only visible in averages which have been used to build up Graph 13 of this work. Otherwise (Text-fig. 28) this intensification of the juxta-epithelial layer's reaction is only seen on a certain stretch of the circumference of the ovule. In the remainder



TEXT-FIGS. 28-30. Acid Proteins in Larger Ovules.

Fig. 28.—Ovary No. 77. Fixative—alcohol. Acid proteins after first chromolysis (Unna's method). Ovule of 5×6 mm. diameter (16×A). An intensification of colour given by pyronine is observed in juxta-epithelial layer. This intensification is limited to a portion of circumference of ovule and ceases almost suddenly. Rest of juxta-epithelial layer in this figure shows same colour intensity as internal theca.

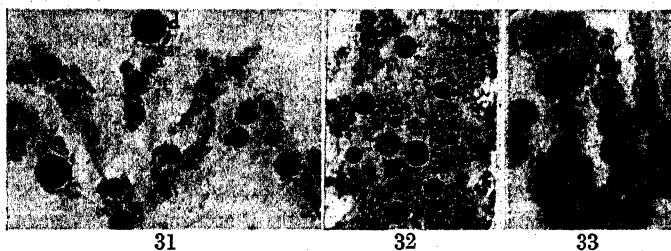
Fig. 29.—Ovary No. 46. Total acid proteins (Unna's method). Ovule of 2.5×2.9 mm. diameter (5×A). Juxta-epithelial layer (J.E.L.) shows a much more intense colour than that of internal theca (I.T.). Reaction is also very intense at level of follicular epithelium. At level of granular cortical layer (G.C.L.), vacuolar reticulum (V.R.), and ovule nucleus, reaction of proteins is rather weak; but is intensified slightly at level of separation (c) of two layers of ovoplasm.

Fig. 30.—Ovary No. 3. Fixative—bichromate of potassium; ferric haematoxylin. Ovule of 340×356 microns diameter (6×D). Between internal theca (I.T.) and follicular epithelium (F.E.) is a layer of very flat cells (a.), intensely coloured, of juxta-epithelial layer.

the juxta-epithelial layer shows the same colouring as the internal theca.

In these ovules there is a concentration at the ovule's surface of substances diffused from the blood, a concentration comparable to what we have seen at the time of our study of the cholesterol.

The follicular epithelium shows an intensification of its colours, compared with smaller ovules. The quantity of globulins has not changed, but the cytoseres are in greater quantity (Diagram 12, Table 17).



TEXT-FIGS. 31-3. Primordial Yolk.

Fig. 31.—Ovary No. 32. Fixative—96% alcohol. Total acid proteins (Unna's method with pyronine methyl-green). Ovule of 5×7 mm. diameter (9×D). With this method periphery of larger globules (*c, d*) of primordial yolk is coloured by methyl-green, while centre is coloured by pyronine, so much the more intensely as the globule has a larger diameter (*c-d*). Smaller globules are only coloured by methyl-green (*a*).

Fig. 32.—Ovary No. 45. Fixative—96% alcohol. Basic proteins. Stain Bordeaux B (Unna's method). Ovule of 3×4 mm. diameter (7×D). With this method periphery appears richer in basic proteins than centre of primordial yolk-globule.

Fig. 33.—Ovary No. 31. Fixative—alcohol. Smear of yolk of an ovule of 3×4 mm. diameter. Feulgen's nuclear reaction. Without Canada balsam. Centre of primordial yolk-globules shows a weakly positive colour.

Ovoplasm.—The granular cortical layer has the appearance and reactions of the ovoplasm of smaller ovules.

The vacuolar reticulum shows the same colouring as the granular cortical layer.

It is not rare to see between the granular cortical layer and the central vacuolar layer a concentration of proteins (Text-

figs. 25 and 29, c). This concentration is not consistent. We have also observed it by other histochemical methods.

**Primordial Yolk.**—In the vacuoles of these ovules yolk is precipitated in the form of rounded globules. The sizes of the globules vary from 3 to 15 microns.

In smaller globules only the blue-green colour of methyl-green is seen (Text-fig. 31, a).

With the increase in diameter of the globules, a substance which takes pyronine very well appears at their centre (Text-fig. 31, b).

The larger globules are made up of a narrow peripheral blue-green band, and with a central red mass, the more red the greater the diameter of the globule (Text-fig. 31, d).

In globules from 4 to 6 microns diameter the colour given by pyronine in the central region of the globule is 0.5; in globules of 15 microns diameter it is 2, 5–8.

The central region of these globules is made up of globulins.

From the point of view of reactions of solubility, the globules of primordial yolk in the hen's egg resemble the vitellin globules of the *Pristiurus* egg. In the latter, Miescher has shown the existence of a central zone, having the solubility of vitellin, and a peripheral zone, differing from vitellin. Miescher has come to the conclusion that the centre of the vitellin globules of the *Pristiurus* egg is made up of vitellin, while at the periphery of these globules the protein forerunner of vitellin is localized.

Is it always vitellin that constitutes the central region of the globules of primordial yolk?

Macallum's methods for showing the presence of iron have given positive reaction at the level of the primordial yolk-globules. But the results obtained from Policard's micro-incineration methods are not sufficiently clear to be able to say with certainty that iron is found in this form of yolk. If, employing the last methods, globules with orange ash are sometimes seen (which indicates the presence of iron) in the majority of globules, the iron, if it exists, is masked by white ash, in which these globules are particularly rich.

In an earlier work (Marza, Marza, and Chiosa, 1932), we have

shown that the histochemical methods of Macallum are subject to many errors, a fact which makes us distrustful of the results obtained by these methods.

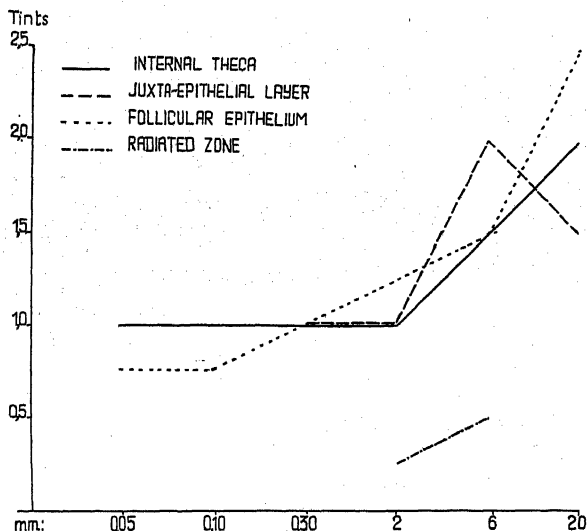


DIAGRAM 13.

Synthetic graph, showing the variations of the acid proteins (globulins and cytosols) in the cytoplasm of the membranes of the hen's egg during the three phases of yolk-formation. Co-ordinate: scale of colours. Abcissae: diameter of the ovules in mm. It is seen that only the follicular epithelium undergoes constant enriching in acid proteins from the beginning of the ovule's development. In the two other membranes the colours of acid proteins remain constant up to when the ovules begin upon the second phase of yolk-formation (ovule of 2-6 mm.). It is also seen that the radiated zone is poorer in acid proteins than the other formations. (Table 17.)

The question of the presence of iron (therefore of haematogen) in primordial yolk-globules remains open. What we can state at present is that at least a part of the vitellin makes its appearance in primordial yolk-globules, and that these globules go through a very interesting series of changes. First, the globules are formed by proteins similar to nucleoproteins. In a later stage of the yolk globules the globulin part of the vitellin makes



its appearance. Finally, in a third stage, the ferric part of the vitellin would be integrated in this molecule. We still make reservations as to its real existence in this last stage at the level of the primordial yolk-globules of the hen's egg.

### Third Phase of Yolk-formation.

Ovules from 6 to 20 mm. Diameter.—Membranes.—The intensity in colour of the internal theca in these ovules is greater than in ovules in slow growth, but the proportion of globulins and cytosols remains the same as before (Table 17 and Diagram 10).

On the other hand, in the juxta-epithelial layer it is the globulins which predominate in the cytoplasm (Diagram 11).

It is not rare to see this layer coloured less intensely than the internal theca (Text-fig. 20); therefore inversely from what one sees sometimes during the second phase of yolk-formation (Text-fig. 29).

The proteins of the follicular epithelium have noticeably increased (Diagrams 12 and 13), but their richness is very variable from one region to another of the same ovule. We are inclined to think that these variations denote that the cells of the follicular epithelium are only laden with proteins so as to yield them to the subjacent yolk (fact also established for cholesterol).

In the case of cholesterol we have noticed that this substance penetrates into the ovules by successive thrusts, that one can follow in different segments in the same ovule. Murray, on the follicular epithelium of large grasshopper's ovules, has also noted variations in the chondriome richness of adjacent cells.

Variations in the amount of proteins in the follicular cells call for identical explanation to that which we have given for the discontinuous penetration of cholesterol through the follicular epithelium.

In the follicular epithelium the proteins seem less attached to the lipoids than in the yolk, which would explain the intensity of globulin reaction at the level of the follicular cells.

The quantity of globulins to be found in the follicular epithelium is greater than that found in ovules of 6 mm. diameter (Table 17). This growth seems in proportion to the enriching

in globulins of the internal theca and the juxta-epithelial layer, under the influence of the ovule's vascularization.

**Yolk.**—As a rule the yolk of these ovules is not very rich in acid proteins (third phase, Diagram 14). The weak reaction

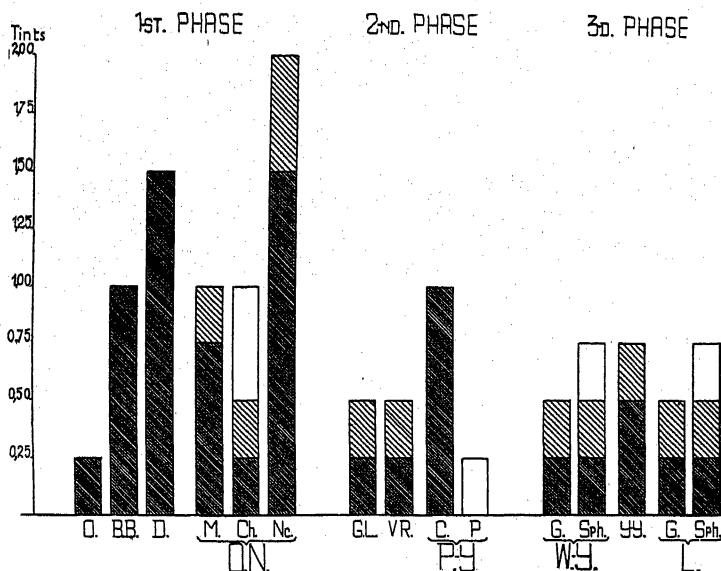


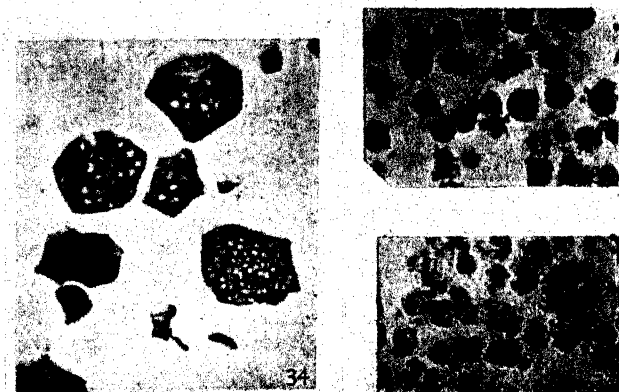
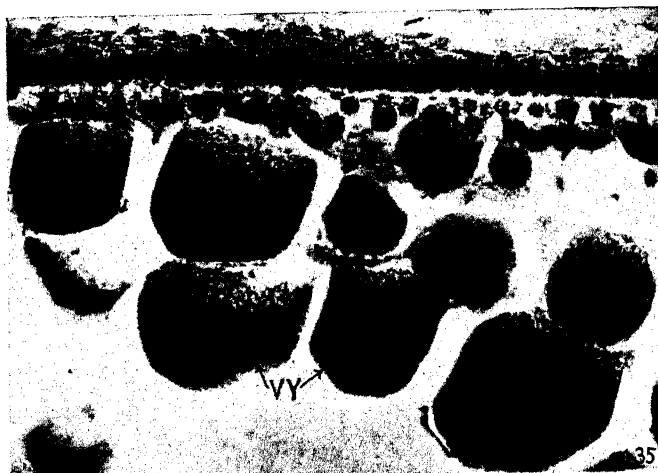
DIAGRAM 14.

The acid proteins in the ovoplasm and the yolk of hens' eggs during yolk-formation (Unna's method for histochemical detection of acid proteins) (Table 18). Co-ordinate: scale of colouring. Same explanation of columns as in Diagram 9.

**First Phase.** O., ovoplasm; BB., Balbiani body; D., densification; ON., ovule's nucleus (M., membrane; CH., chromatin; NC., nucleolus). **Second Phase.** GL., granular-cortical layer; VR., vacuolar reticulum; PY., primordial yolk (C., centre; P., periphery).

**Third Phase.** WY., white yolk (G., ground substance; SPH., spherular inclusions); YY., yellow yolk; L., latebra (G., ground substance; SPH., spherular inclusions).

shown by the yolk can be explained in two ways: (1) by the dispersion of vitellin in the great mass of yolk of ovules in rapid growth; or (2) by the blocking up by means of lipoids of that part of vitellin which reacts like a globulin. In both cases the intensity of the reaction is diminished.



TEXT-FIGS. 34-7. Yolk of Ovules in Rapid Growth.

Fig. 34.—Ovary No. 75. Fixative—96% alcohol. Basic proteins (Unna's Bordeaux B method). Ovule of 22 mm. diameter ( $17\times A$ ). Among globules of yellow yolk of homogeneous aspect are others which show a number of vacuoles inside, vacuoles which are coloured neither by this method, nor by that for showing acid proteins.

Fig. 35.—Ovary No. 45. Fixative—96% alcohol. Basic proteins (Unna's method); stain—Bordeaux B after 48 hours in solution 15 per cent. HCl. Ovule 12 mm. diameter ( $7\times D$ ). Under follicular

In the white cortical yolk one sees certain inclusions which are coloured by methyl-green next to other inclusions whose violet colour is given by a mixture of globulins, pseudoglobulins, and nucleoproteins. In the white yolk one finds the same substances as in the primordial yolk, but arranged differently. One also finds a fundamental substance which is lacking in primordial yolk-globules.

Yellow yolk is not rich in acid proteins. At its level globulins predominate (of the vitellin kind).

In these two forms of yolk (white and yellow), by the method of micro-incineration, one often finds orange ash, which means that in this case the vitellin molecule is complete; but it is very difficult to follow in ovules in rapid growth the stages of building up of the vitellin.

Globules of 'transitional yolk' are found mixed with yellow yolk-globules. These are rich in colourless vacuoles (Text-fig. 34). Konopacka, who in Text-fig. 16 of her remarkable work has typified these globules, has described them as made up of a great number of phosphoprotein globules (see histochemistry of yolk-formation, first part of this work).

The phosphoprotein globules of Konopacka correspond to the colourless vacuoles that we have seen in these globules. In any case, if in these vacuoles one finds proteins, these are neither globulins, cytosols, nor basic-reacting proteins.

The fundamental substance of the 'transitional yolk' globules shows the same colouring as the inter-granular substance of the yellow yolk-globules.

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epithelium (unistratified), a layer of small globules of yolk is observed (wx.). Beneath this layer are large globules of yellow yolk (yx.). Inside them are numerous fine granulations.

Figs. 36 and 37. Ovary No. 75. Fixative—96% alcohol. Basic proteins (fig. 36, Unna's method with Bordeaux B) and acid proteins (fig. 37, Unna's method with pyronine methyl-green). Ovule of 20 mm. diameter ( $6 \times D$ ). Yolk of under-cicatricular region (fig. 37) and of layer D of the latebra (fig. 36). These yolk-globules of very small size are made up of a fundamental substance poor in acid proteins and basic proteins, and of a varying number of spherular inclusions, rich in basic proteins. By method for acid proteins it is seen that certain inclusions are stained by pyronine, and certain others by methyl-green.

In the latebra one finds globules of primordial yolk having the same reactions as ovules during the second phase of yolk-formation; globules of white yolk and globules of 'transitional yolk' which are not distinguishable by histochemical reactions from the globules described above (Text-figs. 36 and 37, Diagram 14).

Therefore, by the method of acid proteins, the same as by the cholesterol method, the construction of the latebra recalls that of ovules during the second phase and during Stage 'A' of the third phase of yolk-formation of the hen's egg.

### Discussion of Results.

Vitellin reacts like a globulin, but one cannot affirm that all globulins that are found in the ovoplasm are vitellin.

During the ovule's growth the ovoplasm is, little by little, impregnated by proteins.

At the beginning of the first phase there are no acid proteins to be seen in the ovoplasm. Later, they make their appearance at the level of the Balbiani body, and in a later stage in the ovoplasm.

During the second phase of yolk-formation the histochemical construction of the ovoplasmic proteins becomes complicated; proteins are seen to appear similar to the nucleoproteins, as well as a part of the vitellin molecule. Finally, during the third phase of yolk-formation, the construction of the proteins is still more complicated. In the white yolk the vitellin, completely formed, is seen next to nucleoproteins. In the yellow yolk one finds vitellin and a pseudoglobulin, which must be livetin.

These changes are probably made under the influence of the enzymes found in the ovoplasm. The existence of many ferments has been observed at this level. Voss has detected the existence of oxidases (frog's egg); Prennant the presence of peroxidases (lamellibranchia, gasteropods); Dulzetto and Parula the existence of glutathione (sea-urchin's eggs); Pennington, Wohlgemuth, Tallarico of lipase (hen's egg); Koga the existence of lecithase, monobutyrase, and thyrosinase (hen's egg); Herlicka of lactase, invertase, and diastase (hen's egg and frog's egg).

It is not illogical to think that ferments govern the synthesis of proteins (vitellin, livetin, nucleoproteins, scleroproteins) in the ovoplasm if one considers the great number of enzymes discovered in the ovoplasm.

Are the stages of vitellin synthesis the same during the rapid growth period of ovules as during the slow growth? The question is not easily answered, considering the speed with which yolk is formed in quick growth ovules. In globules of white cortical yolk one always finds inclusions which are coloured by methyl-green (nucleoproteins) by the side of others which are coloured by pyronine (globulins and cytosols) (Table 18, diagram 14). But the intermediary phases between white and yellow yolk (Stage 'B', rapid growth period) are not sufficiently clear to draw sure conclusions. The stages gone through for the synthesis of vitellin in ovules in rapid growth remain a question that our study has not entered upon. On the other hand, we have observed the stages of this synthesis in primordial yolk of slow growth ovules.

In the appearance of the yolk at the end of the second stage of yolk-formation the hen's ovule nearly resembles holoblastic eggs. This stage once passed through, the morphological and histochemical construction of the yolk, as well as the speed of the ovule's growth, change. The ovule is changed into a meroblastic egg. These changes are so intense and complex that we cannot definitely assert that vitellin is formed according to the same mechanism in ovules of slow growth as those of rapid growth.

**Membranes.**—Examining Tables 17 and 18, and Diagrams 10, 11, and 12, it will be seen that in ovular membranes one finds cytosols in more or less great quantity. In the ovoplasm these proteins are lacking (first and second phase of yolk-formation).

It is still not clearly known if the cytosols (or their nuclear equivalent albumoses) are polypeptids, with a small number of amino-acids, or proteins of a more complex molecule. But it is certain that cytosols are only observed in the case of protein degradation.

Such a process can perhaps be seen at the level of the digestive

tracts where unknown proteins are broken down, afterwards to be resynthesized in the liver or elsewhere.

It seems to us that the ovule shows a new example of an analogous process. The proteins impregnate the ovular membranes in the form of simple protein molecules. Once they have penetrated into the ovoplasm, the proteins are regrouped in larger molecules.

The presence of cytosols in ovular membranes, and their absence in the ovoplasm can be interpreted in this way.

It would appear that the variation in proteins, cholesterol, potassium, &c., in ovular membranes can serve as an indicator of the quantity of substances which diffuse from the blood-vessels to the ovule. These substances once passed out, the blood capillaries percolate first the ovular membranes (internal theca, juxta-epithelial layer, follicular epithelium), and only later pass into the ovoplasm.

During this work, as well as at the time of our study on cholesterol, we have observed that in ovular membranes the substances diffused from the blood-vessels are found in greater quantity than in the ovoplasm. From this excess of proteins and cholesterol, a part only passes through the follicular epithelium and penetrates the ovoplasm. Another part percolates the ovule membrane cells, and a third part is directed to the lymphatic capillaries, from whence it regains the circulation.

The diffusion of substances from the blood to the ovule is made in two stages; first the substances diffused are concentrated at the surface of the ovule, and afterwards a part of these substances reaches the ovoplasm. It probably comes about from an osmotic equilibrium between the ovoplasm and the ovule membranes; the rate of substances diffused into the ovoplasm depends in the first place on the permeability of the follicular epithelium. This would play the part of an ultra-filter, whose construction varies according to the endocrine equilibrium of the hen's organism and the degree of evolution of the egg itself.

We have seen that the diffusion of proteins and cholesterol is made in waves. This diffusion is not synchronous for all

capillaries of the same ovule. The dysynchronism is interpreted, histochemically, by the variations in colour in membranes of the same ovule (Text-fig. 28). Next to a region rich in proteins (acid or basic) one sees another region much poorer in these substances.

The phenomenon of dysynchronic diffusion is not limited to proteins; we have also seen it studying cholesterol.

The richness in proteins of the membranes and the growth of the egg are directly dependent upon the vascularization of the ovule. We have begun the study of this vascularization. From what we have observed up to now, we see that vascularization of ovules is very reduced during the first phase of yolk-formation (Text-figs. 43 and 44). It is not rare to find ovules below 100 microns diameter only possessing a single capillary in their internal theca.

During the second phase of yolk-formation, the vascularization becomes much more important. Numerous capillaries, very dilated, are then seen in the internal theca of ovules (Text-figs. 46 and 47).

Finally, during the third phase of yolk-formation the vascularization of ovules becomes very important. One can see with the naked eye the network of vessels at the ovule's surface.

While the ovarian stroma only shows very reduced vascularization, the blood irrigation of ovules increases continually with the ovule's development. This increase of vascularization explains the concentration of proteins and cholesterol visible in vascularized ovule membranes, and not at the level of the ovarian stroma.

### Permeability of the Follicular Epithelium.

A certain number of observations have shown us that the permeability of the follicular epithelium changes several times during yolk-formation.

We have not had a chance to determine directly the variations in this permeability. But it can be determined indirectly by the variations in chemical composition of the ovoplasm at each phase of yolk-formation of the hen's egg.



The follicular epithelium is a cellular membrane which completely surrounds the ovule. Any substance penetrating the ovoplasm must pass through the follicular epithelium cells. At each stage of the ovule's development the composition of the ovoplasm is dependent upon the permeability of the follicular epithelium. If this permeability changes, the chemical composition of the ovoplasm will also change. That is why we shall consider that one can determine indirectly variations in the permeability of the follicular epithelium by the chemical changes observed in the ovoplasm.

During the ovule's development the chemical composition of the ovoplasm changes three times. One can implicitly deduce that the permeability of the follicular epithelium varies three times during yolk-formation.

In the first part of this work we have described the chemical changes observed in the ovoplasm during the three phases of yolk-formation. These changes can be catalogued thus: during the first phase of yolk-formation the fats penetrate the ovules in very large quantities, while the acid proteins only penetrate in very small quantities into the ovoplasm. The basic proteins, as we shall see later, are found in greater quantity than acid proteins, but their colour does not intensify during the whole first phase of yolk-formation.

It can be established that the characteristic of this phase is the predominance of fat substances over the proteins.

During the second phase of yolk-formation the composition of the ovoplasm changes. Histochemically, it can be proved that there is a greater penetration of acid and basic proteins into the ovule. These proteins give birth to the primordial yolk-globules, whose importance increases towards the end of this phase.

By chemical means, Spohn and Riddle have established that the yolk of these ovules is rich in water and proteins and poor in fat substances. Therefore this phase is characterized by the predominance of proteins over fats.

Finally, during the third phase of yolk-formation of the hen's egg one sees again a modification in the chemical composition of the yolk, which once more becomes rich in lipoids and fats, and poor in water and protein substances (see Table 10, Diagram 7).

If one admits that the changes in chemical composition of the yolk are dependent upon modifications in the permeability of the follicular epithelium, one can ascribe to the latter three variations in permeability during the growth of the hen's egg.

During the first phase of yolk-formation the follicular epithelium is permeable to fat substances; during the second phase it shows greater permeability to protein substances, and during the third phase the follicular epithelium shows once more a greater permeability to fats and lipoids than to proteins.

#### SUMMARY. ACID PROTEINS.

##### (a) Ovule Membranes.

(1) As a rule one sees in ovule membranes (internal theca, juxta-epithelial layer, follicular epithelium) a constant enriching in proteins with acid reaction more evident during the ovule's rapid growth period. This enriching is mostly due to globulins.

(2) The nuclei of the internal theca cells, of the juxta-epithelial layer, and of the follicular epithelium, only undergo minimum variations, which, from the point of view of their intensity, are not comparable to those observed in the cytoplasm of these cells at the time of yolk-formation.

(3) The cytoplasm of the internal theca cells is enriched in acid proteins during the second, and even more so during the third, phase of yolk-formation. But the rate of increase of globulins and cytosols remains the same during the whole of the ovule's development.

(4) The cytoplasm of the juxta-epithelial layer cells is constantly enriched in globulins; these proteins predominate exclusively during the ovule's rapid growth period (third phase of yolk-formation).

The richness of this membrane in cytosols reaches its maximum during the second phase of yolk-formation.

(5) The follicular epithelium undergoes a slow but constant enriching in acid proteins. This enriching is due to globulins.

(6) One frequently sees variation in colour, either at the level of the juxta-epithelial layer (second phase of yolk-formation) or at the level of the follicular epithelium (third phase of yolk-formation) which is shown by a concentration of colour along

a certain length of the circumference of the ovule. This concentration of colour is probably related to the discontinuous passage of protein substances from the vessels to the ovoplasm.

(7) The cytosol, simple protein molecules, are found only at the level of the ovular membranes.

(8) The permeability of the follicular epithelium seems to vary three times: during the first phase of yolk-formation it is more permeable to fat substances, during the second phase to protein substances, and during the third phase the follicular epithelium seems more permeable to lipoids and fats than to protein substances.

#### (b) Ovoplasm Yolk.

First Phase of Yolk-formation.—(9) The ovoplasm remains very poor in acid proteins from the beginning to the end of this long period of the ovule's growth. The weak colour shown by the ovoplasm is due exclusively to globulins.

(10) The Balbiani body is much richer in acid proteins than the ovoplasm. This transitory formation is entirely made up of globulins.

(11) In the ovule nucleus the membrane is made up of globulins. Small quantities of nucleoproteins, globulins, and albumoses are found in the chromatin. The nucleolus has the same histochemical composition as the chromatin, but it is much richer in globulins than the latter.

(12) The chromatin and the nucleolus of the ovule nucleus are only visible at the beginning of this period. They afterwards disappear.

Second Phase of Yolk-formation.—(13) The cortical granular layer and the central reticulum are both poor in acid proteins.

(14) The smaller globules of primordial yolk are made up of nucleoproteins. In larger globules the globulins (vitellin) make their appearance at the centre of the globule. The quantity of globulins increases in direct proportion with the diameter of the vitellin globule; finally, the nucleoproteins form only a narrow band at the periphery of the globule.

Third Phase of Yolk-formation.—(15) During this period the yolk is rather poor in acid proteins.

(16) In yellow yolk-globules the globulins (vitellin) predominate over the pseudoglobulins (livetin).

(17) In white yolk-globules the globulins (vitellin) are in quantity equal to the pseudoglobulins (livetin). In this form of yolk nucleoproteins are also found.

(18) Vacuoles of transitional yolk do not contain acid proteins.

(19) Layer B of the latebra (primordial yolk) and layer D (white yolk) have the same composition as globules of primordial yolk and white yolk of the hen's egg at the end of the second phase of yolk-formation.

## IRON OF THE VITELLIN

### Bunge's Haematogen.

Miescher, and shortly after him Bunge (1882), isolated from the yolk of the hen's egg a protein rich in iron. Bunge, who considered it as the forerunner of haemoglobin, called it haematogen.

Hugounencq and Morel have obtained haematogen from the iron of the vitellin. According to these authors, one finds in a hen's egg 1.44 gr. of vitellin, and 0.045 to 0.050 gr. of haematogen. Therefore haematogen represents 3 to 4 per cent. of the vitellin molecule.

In its turn haematogen can be divided into a protein rich in diamino-acids, and a pigment that Hugounencq and Morel have called haematovine.

The elementary composition of haematogen resembles haemoglobin and nucleins very nearly. There exist, however, great differences between these three molecules, above all between haematogen and nucleins. Between haemoglobin and haematogen the differences are less, which justifies the opinion of Hugounencq and Morel who consider haematogen as a sort of haemoglobin of reserve, not yet differentiated, from whence the embryo must derive the iron for its blood.

### The Iron in the Haematogen.

Bunge and Miescher maintain that iron is found in its organic state. Bunge, who studied the way in which the iron of haematogen reacts with the tests for this metal, came to the conclusion that the link between iron and the protein of haematogen is weaker than in the haemoglobin.

Recently, Levene and Alsberg have shown that the iron of the vitellin is found in the avivitellin acid which they have discovered.

Swigel and Posternak, splitting vitellin by tryptic digestion, have obtained three polypeptides: ovotyrines  $\alpha$ ,  $\beta$ , and  $\gamma$ .

Iron is localized in ovotyrine  $\beta_2$ , which would have the composition of the avivitellin acid of Levene and Alsberg. In the ovotyrine iron is found in its ferric state, and reacts quickly with sulfo- and ferro-cyanide.

In 1929 Hill, with the help of the dipyrindyle reaction, finds that 95 per cent. of the iron in the yolk of the hen's egg is in its inorganic state. McFarlane, 1932, finds that the iron of the vitellin is in loose organic combination.

### Iron in other Kinds of Eggs.

The presence of iron in the vitellin of the hen's egg has incited many authors to seek this metal by chemical methods in the yolk of very different kinds of eggs.

Iron has been found in eggs of a certain number of Invertebrata (sea-urchin, cuttlefish, &c.), as well as in the eggs of amphibians, fishes, &c.<sup>1</sup>

### Histochemical Detection of Iron in the Yolk.

Schneider was the first author who, with the help of the Prussian blue reaction, showed the presence of iron in the yolk of the hen's egg (Wassermann).

According to Bunge and Schneider, all the elements of the egg yolk contain iron. This is not Smiechowski's opinion, who,

<sup>1</sup> For details on this question see Joseph Needham's 'Chemical Embryology', where the problem of the existence of iron in the yolk, the authors and the kinds studied, and the quantity found in each case, are very well expressed and tabulated.

in 1892, applying Prussian blue reaction to the study of yolk, came to the conclusion that only white yolk gives iron reaction.

With these contradictory results, in 1910, Wassermann once more took up the question of localization of iron in the yellow of the hen's egg. He has used several methods of histochemical detection of iron, and has studied not only the localization of iron in the yolk but also the comparative worth of the different processes proposed for histochemical detection of this metal.

Wassermann's study is much more complete than that of Smiechowski.

According to Wassermann, the white yolk, quite as much as the yellow yolk, contains iron, but the white yolk gives the more intense reaction. The latebra and the yolk of the nucleus of Pander would react in the same manner as the white yolk.

In 1932, in collaboration with Mme Marza and Chiosa, we have studied the localization of iron during the development of the hen's egg, using Macallum's histochemical methods as well as Policard's micro-incineration method.

### Methods.

For histochemical detection of iron we know a series of methods, now old-fashioned, of Macallum's, and a very up-to-date method of Policard's.

Macallum's histochemical methods use the colour reactions that iron gives with ferro- and ferricyanide of potassium, with rhodanate of ammonium, sulphide of potassium, and ferric haemotoxylin. This author also established the techniques that it is necessary to follow to detect organic and inorganic iron.

Policard's method recommends the incineration of segments in an electric oven up to the complete disappearance of the carbon. Thus one obtains the calcined skeleton of the section, where it is easy to distinguish on a black ground all details necessary for the interpretation of the section.

The salts of Ca, Mg, P, Si, give white ash, more or less brilliant. Only iron gives red oxides (if it is found in great quantity) or orange (if the iron is mixed with the ash of other elements). The salts of K, Na, and chlorides volatilize, giving no ash in this method.

Macallum's methods have aroused much criticism as we have shown at some length in our 1932 work on iron (pp. 214-16). We have come to the conclusion that Macallum's methods lend themselves to errors that Policard's method avoids. For this reason we have given preference to results obtained by this last method, as to localization of iron in the yolk.

### Material.

As material for study, we have used fifteen hens' ovaries fixed in alcohol at 96°.

On a series of sections (cut at 8 microns) we have carried out Macallum's methods and Policard's for the histochemical detection of iron.

Policard's micro-incineration method has allowed us to draw a certain number of relative conclusions as to the richness in fixed ash of the hen's egg and its membranes during yolk-formation, as described in the first part of this work.

### Results.

Iron is absent from the ovarian stroma, from the internal theca, the juxta-epithelial layer, the follicular epithelium, and the radiated zone of ovules during the whole of the latter's development.

In the internal theca and in the ovarian stroma iron is only seen at the level of the red blood corpuscles, where it is very easily visible.

Iron does not give positive reaction either in the ovoplasm or in the Balbiani body, or in the nuclei of the ovule. As regards these last, we cannot agree with Macallum, who shows iron at the level of the nucleus of batrachian ovules.

During the second phase of yolk-formation one does not find iron either in the granular cortical layer or in the central reticulum of ovules.

The primordial yolk found in these ovules sometimes shows orange ash, which signifies that at least some of these globules possess a small quantity of iron.

The possibility of synthesizing every molecule of vitellin exists then from this phase of development of the hen's egg,

but this possibility seems limited to a small number of globules; that is to say, it is only outlined during this stage. At least, that is the conclusion to which one is led, using Policard's micro-incineration method.

What makes the reading of results difficult by this method is the great quantity of ash that the primordial yolk-globules possess. If these globules contain traces of iron, the red colour of this ash can be masked by white ash of other salts. To detect traces of iron it would be necessary to use Policard's histospectograph.

The results we have obtained by the micro-incineration method contrast with the results reached by us, on the same material, by Macallum's methods with ferrocyanide of potassium and sulphide of ammonium. By these two last methods almost all globules of primordial yolk show a positive reaction. We interpret these results as being due to traces of iron that the globules have absorbed either from reagents or from the glass slide on which the sections were stuck during the sojourn of the section in the sulphuric alcohol solution.

If one compares the results obtained by Policard's method with those obtained by Unna's method of pyronine methyl-green, it is seen that by this last method globulins are frequently found at the centre of the primordial yolk-globules. By the method of micro-incineration iron is only rarely found at the level of these globules. If in primordial yolk-globules vitellin gives the reaction of globulins, and haematogen the reaction of iron, one can suppose that the molecule of vitellin is not completely synthesized at the time of this phase; the rarity of the iron reaction shows that haematogen is not present in all these globulins.

From these observations we have arrived at the hypothesis that vitellin is formed in stages.

During the third phase of yolk-formation the globules with yellow ash are not very numerous at the beginning, but more abundant towards the end, when it is not unusual to find globules with clear pink ash, or even red.

In this period the molecule appears completely formed, or in any case to have acquired its iron.



In the laid egg the globules of yellow and white yolk show the presence of orange ash. It is rather rare to find globules with clear red ash.

It is difficult to establish a comparison between the richness in iron of the yellow and the white yolk. The yellow yolk-globules, during micro-incineration, swell and burst in pieces, which makes it particularly difficult to compare them with globules of cortical white yolk.

We can state, however, that the two forms of yolk contain iron (that is to say haematogen), and from this point of view we confirm the suppositions of Bunge and Schneider, as well as the researches of Wassermann.

### Conclusions.

- (1) The ovular membranes do not contain iron in their cells.
- (2) No element of the ovoplasm shows the presence of iron at its level during the first phase of yolk-formation.
- (3) A small number of primordial yolk-globules show the presence of iron (that is to say, haematogen) at their level.
- (4) The white yolk-globules, as well as those of yellow yolk, contain iron.

### B. DETECTION OF PROTEINS WITH BASIC REACTION (Plastine).

#### Method.

To detect proteins of basic reaction we have used Unna's Bordeaux B method.

Bordeaux B is an acid stain (naphthylamine-azonaphthol-disulphonic acid) prepared for the first time by Unna, and known in histological technique by the name of 'Bordo' or Bordeaux B. According to Unna, the Bordeaux B shows plastine (protein of basic reaction) due to the disulphonic and phenolic groupings of its molecule.

The Bordeaux B method is one of the simplest to perform. The sections (paraffin removed and hydrated) remain 3 minutes in a Bordeaux B solution of 0.5 per cent. Wash with tap-water. Colour for 3 minutes with Unna's alum haematin. Wash again with tap-water. Dehydration, xylol, Canada balsam.

One can dispense with haemalum staining. The Bordeaux B coloration is amply sufficient for a microscopic examination of the section.

Since the Bordeaux B solution loses its colouring power quickly, in order to have comparable results we have used the same solution of Bordeaux B for only two weeks at the most.

In the appraisal of results we are aided by a scale of colour of six tints. Unna has affirmed that under the action of hydrochloric acid, the colour of the section is intensified. According to Unna this intensification is due to the freeing of basic proteins joined to the acid proteins.

One can therefore (according to Unna) show the free basic proteins and the combined basic proteins.

### Plastine.

Plastine is not a well-known and classified protein. Described by Zacharias, it has formed the subject of a very small number of researches, which moreover have come to very contradictory results.

The majority of authors do not consider it as a very definite chemical entity, others think of it as a scleroprotein.

Unna maintains that plastine is a basic protein which comes into the composition of every cell.

Kiesel, who in his book, 'Chemie der Protoplasmas', has devoted a very documented study to plastine, comes to the conclusion that, up to the present, we cannot specify the place that plastine must occupy among proteins.

The same ignorance reigns as regards the scleroproteins of the hen's egg. Of these, we only know that they make up the vitelline membrane of the egg (J. Needham and D. Needham).

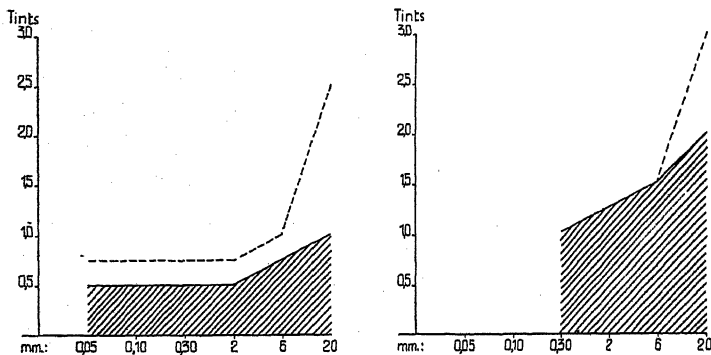
For these reasons we will not insist on the value of Unna's Bordeaux B method.

In the hope that one day the question of plastine or scleroproteins of the yolk will be determined, we have used this reaction, which, although it does not show a well-defined protein, will at least show the localization of basic functions of ovular proteins, and from this point of view the reaction to Bordeaux B can render us service.

## Results.

## First Phase of Yolk-formation.

Ovules from 50 to 100 microns Diameter.—The follicular epithelium is rich in basic proteins, but the internal theca is rather poor.



DIAGRAMS 15 AND 16.

The average colours of basic proteins in ovular membranes during the growth of the hen's egg (Unna's method for histochemical detection of basic proteins) (Table 19). Ordinate: scale of colouring; abscissa: diameter of ovules in mm. Hatching: free basic proteins; white: combined basic proteins.

Diagram 15 represents the basic proteins of the internal theca; Diagram 16 of the juxta-epithelial layer.

Hydrolysis, made with the help of HCl 15 per cent., liberates a certain quantity of basic proteins in the internal theca, and the Balbiani body. The follicular epithelium and the ovoplasm seem not to contain combined basic proteins (Diagram 18).

The basic proteins of the Balbiani body are probably joined to the globulins.

Ovules from 100 to 300 microns Diameter.—The colours of the internal theca of the follicular epithelium and of the ovoplasm have not changed (Table 19 and Diagrams 15, 16, and 17). The Balbiani body has disappeared.

At this stage, by methods for detection of fats, one observes

a layer of fat globules at some distance from the periphery of the ovule (Text-fig. 2). By the Bordeaux B method no variation in colour is seen at this level.

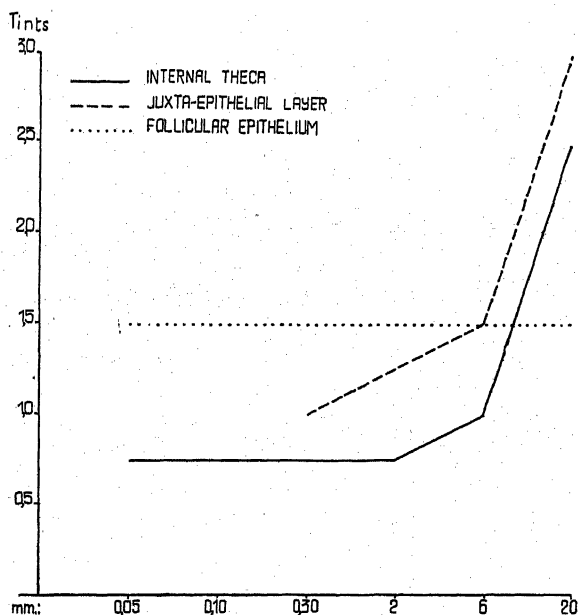


DIAGRAM 17.

Synthetic graph, showing the variations in basic proteins (free and combined) in the membranes of the hen's egg, during the three phases of yolk-formation (Table 19). Co-ordinate: scale of colouring. Abcissae: diameter of the ovules in mm. It is seen that the colours of the follicular epithelium do not vary during the whole of the ovule's growth. The internal theca is only enriched in basic proteins from the second phase of yolk-formation (ovules of 2-6 mm. diameter). The internal theca as well as the juxta-epithelial layer is considerably enriched in basic proteins during the third phase of yolk-formation (ovules above 6 mm. diameter).

Ovules from 300 to 2,000 microns Diameter.—The internal theca and the follicular epithelium have kept the same colours as around smaller ovules (Table 19, Diagrams 15 and 17).

The ovoplasm is a little richer in basic proteins than before.

### Second Period of the Ovule's Growth.

Ovules from 2 to 6 mm. Diameter.—The internal theca as well as the juxta-epithelial layer are enriched in basic proteins (Diagrams 15, 16, and 17, Table 19). The latter layer always remains richer in plastine than the former (Diagram 17).

The follicular epithelium has kept its initial richness in basic proteins (Diagram 18).

The cortical granular layer has the same colour as the granular ovoplasm of smaller ovules.

In primordial yolk-globules the centre of the globules is less rich in basic proteins than the periphery (Text-fig. 32, Diagram 18), therefore inversely of what we have seen in the case of acid proteins (Text-fig. 31, Diagram 14).

If Bordeaux B is a test for sclero-protein, its concentration at the periphery of the primordial yolk-globule would explain the particularly great resistance of these globules to mechanical factors (compression), a fact that we have noted while making a smear of yolk. The yellow yolk-globules (at the periphery of which one sees no concentration with Bordeaux B) burst at the least pressure, allowing a swarm of very fine granulations to escape from their interior.

### Third Phase of Yolk-formation.

Ovules from 6 to 20 mm. Diameter.—In the cells of the internal theca and the juxta-epithelial layer one observes an enriching in plastine in proportion to what one sees in the same layers of smaller ovules (Diagrams 15 and 16, Table 19).

During this period the juxta-epithelial layer is still richer in basic proteins than the internal theca (Diagram 17, Table 19).

In these two ovular membranes the quantity of combined basic proteins is rather large (Diagrams 15 and 16).

The follicular epithelium has not changed its composition. At its level combined basic proteins are not seen.

Yolk.—The yellow yolk globules contain fine granulations (Text-fig. 35). These granulations (which are not seen by the acid protein method) were observed a long time ago in the yellow yolk-globules.

Numerous histochemical reactions have shown us that substances shown in the yolk are concentrated at the level of the granulations of yellow yolk-globules (V. Marza, L. Chiosa, N. Feldman).

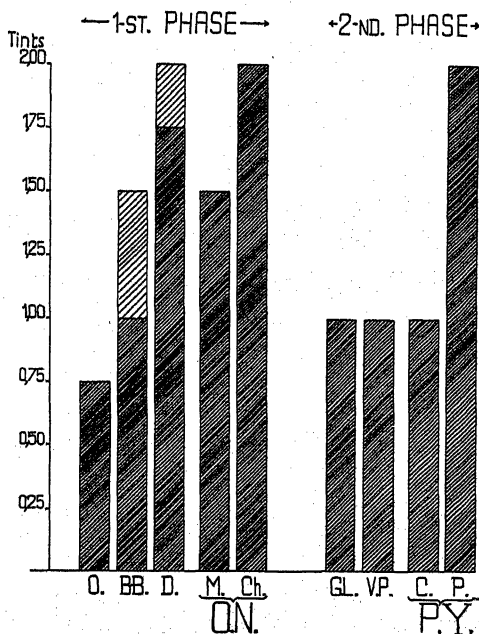


DIAGRAM 18.

The richness in basic proteins (free and combined) of the ovule nucleus, the ovoplasm, and the yolk during yolk-formation of the hen's egg; Unna's method for testing for basic proteins (Table 20). Read the same way as Diagram 14. Co-ordinate: scale of colouring. (See also diagram 19.)

By the Bordeaux B method it is seen that the granulations of the yellow yolk-globule, show a more intense colour (tint 2) than the intergranular mass (tint '1') of the globule (Text-fig. 35, *yy.*, Diagram 19).

It is observed in yellow yolk-globules that HCl 15 per cent. frees a great quantity of basic proteins, which intensify the colouring of these globules.

White yolk-globules are made up of inclusions having

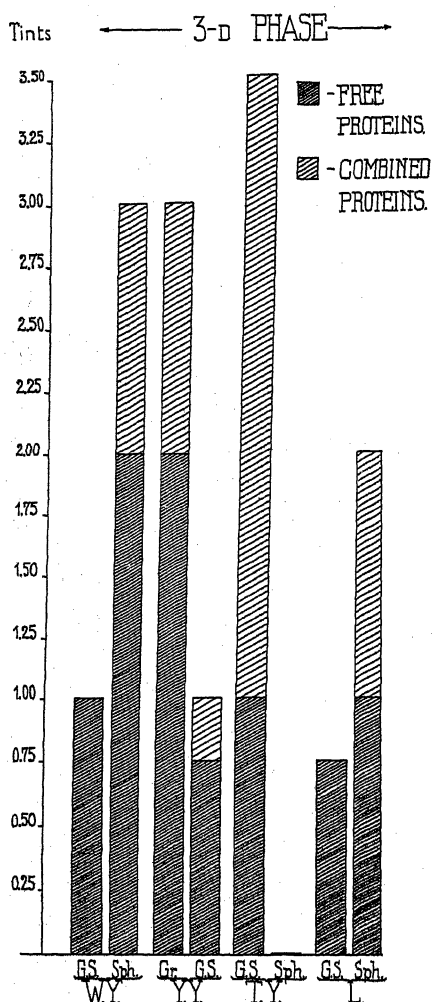


DIAGRAM 19.

For description see Diagram 18.

intensely positive reaction, plunged in a fundamental substance which takes Bordeaux B a little.

Transitional yolk-globules are composed of vacuoles (which

do not colour with Bordeaux B) and a fundamental substance which is intensely coloured (Text-fig. 34; *TX.*, Diagram 19).

TABLE 19.

The average colours of the basic proteins in ovular membranes during yolk-formation (hen's egg). *F.*, free basic proteins; *C.*, combined basic proteins, and freed by the help of HCl 15 per cent.

<i>Diameter of Ovules (in mm.).</i>	<i>Internal Theca.</i>		<i>Juxta-epithe- lial Layer.</i>		<i>Follicular Epithelium.</i>		<i>Radiated Zone.</i>	
	<i>F.</i>	<i>C.</i>	<i>F.</i>	<i>C.</i>	<i>F.</i>	<i>C.</i>	<i>F.</i>	<i>C.</i>
0.05 . . .	0.5	0.25	..	..	1.5	0	..	..
0.1 . . .	0.5	0.25	..	..	1.5	0	..	..
0.3 . . .	0.5	0.25	1.0	0	1.5	0	..	..
2.0 . . .	0.5	0.25	1.25	0	1.5	0	1.0	0.5
6.0 . . .	0.75	0.25	1.5	0	1.5	0	..	..
20.0 . . .	1.0	1.5	2.0	1.0	1.5	0	..	..

The latebra globules and those of the nucleus of Pander (Text-fig. 36) have the same composition as white yolk-globules (layer D of the latebra), or primordial yolk (layer B of the latebra).

In layer D globules the small inclusions of the globule are rich in basic proteins, while the fundamental substance is poor in them.

### Discussions.

As a rule, the ovoplasm of small ovules has much more affinity for Bordeaux B than for pyronine.

In different kinds of ovules certain authors (Dubuisson, Hirschler, Parat) have noted an acidophil reaction at the beginning of the ovule's development, which gives way, little by little, to a basiphil reaction. We have seen nothing analogous in the ovoplasm's reaction at the beginning of this period.

If Bordeaux B shows scleroprotein, this does not seem to have the same importance for the general metabolism of the ovule as vitellin and livetin.

The basic proteins seem to take a passive mechanical part in the ovule, being elements indispensable to the construction of the ovoplasm and yolk-elements.



TABLE 20.

The average colours of basic proteins, stainable with Bordeaux B, in the ovoplasm and yolk, during yolk-formation of the hen's egg. F., free basic proteins; C., combined basic proteins.

	First Phase.	F.	C.
Ovoplasm . . . . .		0.75	0
Balbani body . . . . .		1.0	0.5
Densification . . . . .		1.75	0.25
Ovule nucleus.			
Membrane . . . . .		1.5	0
Chromatin . . . . .		2.0	0
	Second Phase.		
Granular cortical layer . . . . .		1.0	0
Intervacuolar reticulum . . . . .		1.0	0
Primordial yolk.			
Centre . . . . .		1.0	0
Periphery . . . . .		2.0	0
	Third Phase.		
White yolk.			
Fundamental substance . . . . .		1.0	0
Inclusions . . . . .		2.0	1.0
Yellow Yolk.			
Fundamental substance . . . . .		0.75	0.25
Granulations . . . . .		2.0	1.0
Transitional yolk.			
Vacuoles . . . . .		0	0
Fundamental substance . . . . .		1.0	2.5
Latebra (Layer D).			
Inclusions . . . . .		1.0	1.0
Fundamental substance . . . . .		1.0	0

### Conclusions.

(1) The colour of basic proteins in the membranes is intensified during the two last phases of yolk-formation.

(2) The colour of basic proteins does not change during the whole of yolk-formation in the follicular epithelium cells.

(3) During the first phase of yolk-formation the colour of the ovoplasm does not change.

(4) In primordial yolk-globules the periphery of the globule is richer in basic proteins than the centre.

(5) The fine granulations of yellow yolk-globules, as well as the inclusions of white yolk-globules, are richer in basic proteins

than the fundamental substance of these two types of vitellin globule.

(6) The vacuoles of transitional yolk-globules show negative reaction, the fundamental substance an intensely positive reaction.

(7) The reactions of the latebra layers are identical with those of white yolk and primordial yolk.

(8) In yellow yolk and white yolk a great quantity of combined basic proteins are found.

### C. NUCLEOPROTEINS. THYMONUCLEIC ACID.

The presence of nucleoproteins in eggs has raised very difficult discussions relative to the way in which these conjugated proteins are composed.

In a remarkable series of researches J. and D. Needham have demonstrated that eggs of aquatic development are much richer in nucleoproteins than eggs developed on land.

Aquatic eggs synthesize nucleoproteins at the time of their yolk-formation. Terrestrial-developed eggs synthesize these proteins only after fertilization, which explains why aquatic eggs are rich in nucleoproteins, while terrestrial eggs are not.

In the hen's egg the nucleoproteins are in very small quantity. They do not go beyond 2 mgr. in the laid egg (Plimmer and Scott, Heubner and Reeb, Fridericia, Sendju and Mendell).

In 1933 Brachet demonstrated that nucleoproteins, accumulated in large quantity in the sea-urchin's egg, are not made up of thymonucleic acid. The latter is synthesized only during development by a transformation of the nucleic acids into thymonucleic acid.

As for the composition of thymonucleic acid after fertilization, there exist great differences between terrestrial eggs and aquatic eggs. In the former (e.g. hen's egg) every particle must be synthesized in the thymonucleic acid, while in the latter, a synthesis, properly so-called, does not take place, but a transformation from nucleic acids of the yolk to thymonucleic acid.

### Method.

To show nuclear substances, histochemistry has only two methods; one, methyl-green, for showing nucleoproteins, the

other colourless fuchsin (Feulgen's nuclear reaction for showing thymonucleic acid).

In a recent work<sup>1</sup> we have discussed the value of each one of these two methods. We shall not return to these questions here.

### Nuclear Reaction in Eggs.

The nuclear reaction has been applied to the study of many kinds of ovules of Invertebrata and Vertebrata.<sup>2</sup> In the hen's egg it has been studied by V. Marza and E. Marza (1934). For the latter kind the interest of knowing the variations in thymonucleic acid is so much greater, as hens have a peculiar behaviour as regards the synthesis of thymonucleic acid.

From works of Brachet and other authors, who have applied this reaction to the study of yolk-formation, it results that in a general way nuclear reaction becomes negative in the nuclei of ovules during growth. During maturation of ovules, nuclear reaction again becomes positive at the level of the chromosomes.

J. Brachet attributes the negative reaction of the oocyte's nucleus to a chemical transformation of thymonucleic acid during yolk-formation. He refutes Feulgen's hypothesis, which attributes negative reaction to the dilution of thymonucleic acid in the nuclear mass, which increases during the ovule's growth.

### Results.

In the section on acid proteins we have described the histochemical composition of nuclei of the ovular membranes (Tables 15 and 16, Diagram 9), as well as in the nucleus of the ovule (Table 18, Diagrams 14 and 18).

In this section we shall describe results obtained applying nuclear reaction to the same material.

In the nuclei of ovular membranes the nuclear reaction is only positive at the level of the chromatin and nucleolus. Therefore there is perfect concordance between results obtained by methyl-green and those obtained by nuclear reaction.

In the ovule nucleus chromatin gives weak reaction, in smaller ovules.

<sup>1</sup> V. Marza and E. Marza (1934).

<sup>2</sup> For the bibliography of this question see J. Brachet's article (1933).



TEXT-FIGS. 38-9. Nuclear Reaction in Small Ovules.

Fig. 38.—Ovary No. 37. Fixative—acetic sublimate. Feulgen's method, nuclear reaction ( $7\times A$ ). View of part of ovary. It is seen that reaction is weaker at level of nuclei of ovarian stroma, negative at level of ovule nucleus, and intense at level of nuclei of ovular membranes.

Fig. 39.—Same ovary. Ovule of  $104\times 196$  microns diameter. Reaction is positive at level of nuclei of internal theca, juxta-epithelial layer with very elongated nuclei and follicular epithelium; reaction is negative at level of ovule nucleus. In the follicular membranes, nuclear reaction is positive at level of chromatin and nucleolus.

The reaction of the ovule nucleus weakens quickly and becomes negative in ovules from 100 to 150 microns diameter, while the nuclei of membranes show intensely positive reactions during the whole of the ovule's evolution (Text-fig. 39).

In our scale of colouring the reaction shown by an ovule nucleus of 50–80 microns diameter is eight times less intense than the reaction shown by the nuclei of the follicular epithelium.

Nuclear reaction is of a weaker intensity at the level of the ovarian stroma. The differences between the stroma and the membranes are clearly seen in Text-fig. 38.

Ovoplasm.—The ovoplasm shows negative reaction during the first period of the ovule's growth.

The Balbiani body gives negative reaction by this method (Text-fig. 39).

During the second phase of yolk-formation the granular cortical layer and the vacuolar reticulum show negative reaction (Text-fig. 40).

Primordial yolk-globules show a weakly positive reaction at their centre which does not correspond to localization with methyl-green on these globules (Text-fig. 33). Moreover, by plasmas reaction one sees again a weak reaction in the central region of the primordial yolk-globules.

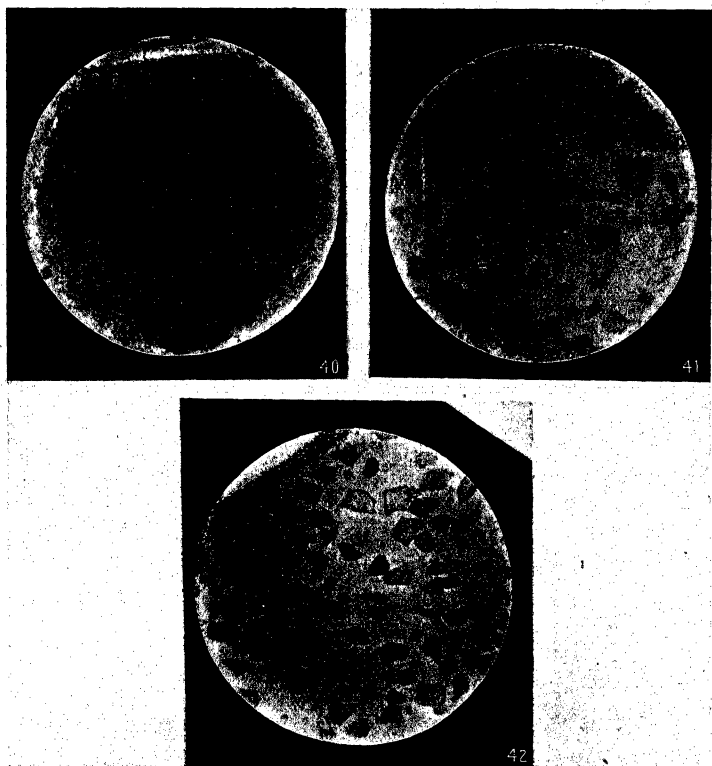
We do not put down the weak positive reaction observed in the primordial yolk-globules to the thymonucleic acid.

A certain number of substances react with fuchsin-sulphide reagent (used in Feulgen's nuclear reaction) colouring it.<sup>1</sup>

It is probably the alkalis (potassium) which provoke the appearance of a positive reaction in primordial yolk-globules. In fact, studying the histochemical localization of potassium in the hen's egg yolk, we have observed a particularly intense reaction of potassium at the level of primordial yolk-globules (Text-fig. 7).

During the third phase of yolk-formation the white yolk-globules give a very weak nuclear reaction. In this case the

<sup>1</sup> Alkali; acetone; aliphatic cetenes; lipoids; brom; buffered salts of strong alkali (acetates; phosphates, etc.); amino-oxides; oxidative catalytic systems (Lison, 1932).



TEXT-FIGS. 40-2. Nuclear Reaction.

Fig. 40.—Ovary No. 36. Fixative—96% alcohol. Nuclear reaction (Feulgen's method). Ovule of 2 mm. diameter ( $7 \times D$ ). Reaction is negative in ovoplasm and vacuolar reticulum, nuclear very weakly positive at level of primordial yolk-globules, and intense at level of follicular epithelium nuclei.

Fig. 41.—Ovary No. 75. Fixative—96% alcohol. Nuclear reaction (Feulgen's method). Ovule of 23 mm. diameter ( $7 \times A$ ). At level of yellow yolk-globules reaction is negative; it is positive at level of ovular membrane nuclei.

Fig. 42.—Same ovary. Same ovule. Section consumed in alcohol and ether in a Soxhlet apparatus ( $7 \times A$ ). After treatment in alcohol and ether negative reaction of yolk becomes weakly positive, thus confirming Brachet's observations.

localization with this reaction corresponds to the localization with methyl-green. By these two reactions a certain number of inclusions is shown in which these globules are particularly rich. Another part of these inclusions does not give positive nuclear reaction. The yolk of Layer 'D' of the latebra reacts identically with white yolk.

The yellow yolk-globules show negative nuclear reaction or sometimes an extremely weak reaction (Text-fig. 41).

### Discussion of Results.

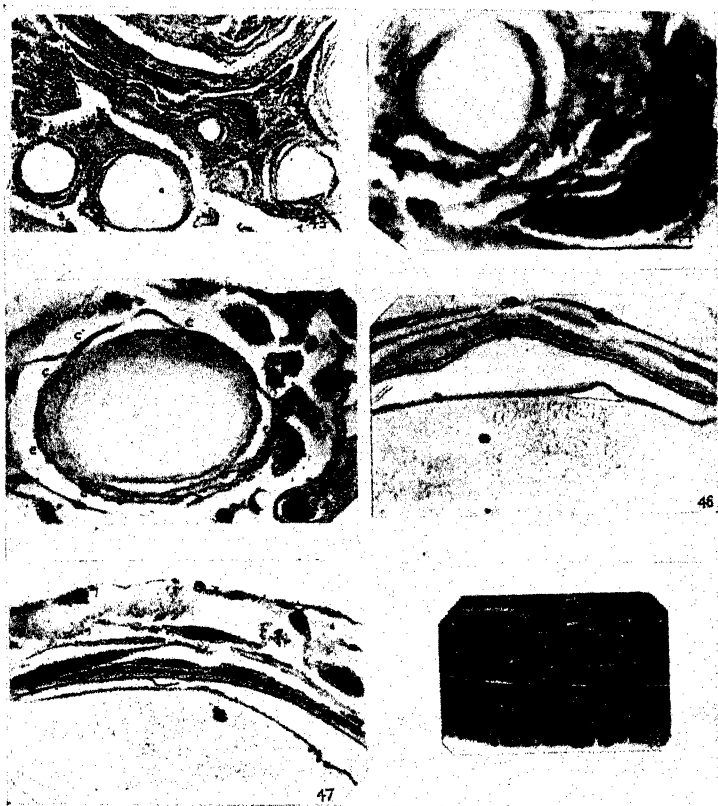
In the egg of the toad *Discoglossus* Hope Hibbard has observed positive reaction at the level of the Balbiani body. According to this author the reaction would be due to a simpler form of thymonucleic acid, which comes from the nucleus and spreads over into the ovoplasm. In this way Hope Hibbard explains the negative reaction observed in the nuclei of ovules during growth.

Brachet refutes Hope Hibbard's hypothesis. The Balbiani body in all the kinds of ovules which he has studied does not show a positive nuclear reaction. In our turn we have observed in the hen that the plasmal reaction seen at the level of the Balbiani body disappears by hydrolysis, which argues against the presence of thymonucleic acid in this place.

As regards the yolk, we have already expressed our relative opinions as to the positive reaction that one observes in primordial yolk-globules and white yolk-globules.

Fridericia supposes that the small quantity of nucleoproteins that one finds in the hen's egg is localized in the blastodermic vesicle (that is to say the nucleus) of the hen's egg.

From the work of van Durme it has resulted that the germinating vesicle of the hen's egg is particularly small (see nuclear changes during yolk-formation in the first part of this work). Given the very small size of the germinating vesicle of the ovule and above all of the chromosomes, it seems to us hardly probable that these latter elements are the only depositaries of the nucleoproteins. Our histochemical researches have allowed us to see that the white yolk of the latebra and the white cortical yolk both contain a very small quantity of nucleoproteins.



TEXT-FIGS. 43-8. Blood supply of Ovules.

Fig. 43.—Ovary No. 54. Reaction of haemoglobin (Lison's method) ( $5\times A$ ) (haemoglobin is in black). General view of part of ovary. Big blood-vessels are numerous in stroma, but rare in ovule membranes.

Fig. 44.—Enlarged part of preceding figure. Ovule of  $105\times 107$  microns diameter ( $5\times D$ ). Next to ovule is a large blood-vessel (in black); but in internal theca of this ovule there are only small capillaries.

Fig. 45.—Same ovary. Ovule  $300\times 412$  microns diameter (oc. comp.  $8\times A$ ). Ovule near hilum of ovary; yet in internal theca of this ovule there are only small capillaries (in 'c').

Fig. 46.—Ovary No. 54. Reaction with haemoglobin (Lison's



## Conclusions.

- (1) The nuclei of ovular membranes show an intensely positive nuclear reaction at the level of the chromatin and the nucleolus.
- (2) The nuclei of the ovarian stroma show a less intense nuclear reaction than that of the nuclei of ovular membranes.
- (3) The ovule nucleus shows a very weak nuclear reaction in very small ovules. This reaction becomes negative in larger ovules.
- (4) Neither the ovoplasm, nor the Balbiani body, give a positive nuclear reaction.
- (5) The reaction that one observes at the centre of the primordial yolk-globules does not seem to be due to the presence of thymonucleic acid at this level.
- (6) In the white yolk-inclusions one sees a very weak nuclear reaction.

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method). Ovule of  $4 \times 4.5$  mm. diameter (second phase of yolk-formation) ( $5 \times A$ ).

Fig. 47.—Same ovary. Another part of the ovule of fig. 46 ( $5 \times A$ ). While part shown in fig. 46 is poor in blood-vessels, that in fig. 47 is very rich in them. Vascular network is not spread uniformly over circumference of such an ovule.

Fig. 48.—Ovary 7. Fixative—bichromate of potassium. Stain—ferric haematoxylin. Ovule of  $3 \times 3.5$  mm. diameter ( $5 \times D$ ) (second phase of yolk-formation). During this phase blood capillaries (in black) come very near to follicular epithelium (FE) from which they are only separated by one or two layers of very flat cells (of juxta-epithelial layer).

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# The Vascular System of *Octochaetus thomasi*

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With Plates 8 and 9.

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## INTRODUCTION.

THE study of the vascular system in earthworms has attracted many authors, but no detailed account has been published of the system in New Zealand earthworms or indeed in any of the Acanthodrilinae. The paper which follows deals with the relations of the blood-vessels and the circulation of the blood in *Octochaetus thomasi* Beddard, an earthworm belonging to this family. No account is given of the ultimate distribution of the vessels within the body-wall and in the nephridia since this is essentially similar in all earthworms, and has been the subject of careful description by Benham (5), Harrington (9), and Bahl (1 and 2).

The work was carried out in the Zoology Department of the University of Otago under the direction of Professor Wm. B. Benham, F.R.S., to whom I take this opportunity of expressing my thanks for his helpful criticism and advice both in the progress of the work and in the preparation of this manuscript.

## PREVIOUS WORK.

It appears that the first zoologist to give a fairly correct account of the course taken by the blood in an earthworm was Antoine Dugés,<sup>1</sup> who in 1828 (p. 298) observed that the 'pulse' of the dorsal vessel sent the blood from behind forward, where it enters the 'plexiform' vessels (i.e. lateral hearts) so as to reach the ventral vessels, of which he recognized both the 'sub-intestinal' and the 'subneural'. Further, he was led to believe that the blood passed upwards through the commissural vessels (his 'abdomino-dorsal branches') and so entered the dorsal vessel. These facts he established by cutting the worm into two and noting the flow of blood from the cut vessels in each half. The earthworms studied were, of course, *Lumbricus* and allied genera.

Beddard (3 and 4) gives a brief account of the major blood-vessels of *O. multiporus* and *O. thomasi* in his descriptions of the species. He describes the dorsal vessel as completely double behind the gizzard. On this evidence, Stephenson (11) describes the dorsal vessel of earthworms as varying from single, incompletely double—where the two limbs unite at the septa—to a completely double condition, *O. multiporus* being taken as an example of this last type.

Benham (6) describes the major blood-vessels of the cephalized region of *Maoridrilus uliginosus*, noting the double nature of the dorsal and supra-intestinal vessels, and the segmental position of the heart and lateral vessel.

Bourne (7 and 8) described the vascular system and the circulation in *Megascolex caeruleus*. Of anatomical interest was his description of the hearts as both latero-intestinal, arising dorsally from both dorsal and supra-intestinal vessels, and lateral, arising from dorsal vessel alone. Bourne studied the course of the blood by observing the contractions of the vessels, the arrangement and disposition of the valves, the condition produced by pinching the vessel between fine forceps, and the bleeding of severed vessels. He agreed with previous authors

<sup>1</sup> Dugés (1828), "Recherches sur la Circulation, la Respiration et la Reproduction des Annélides Abranches", 'Annales des Sciences naturelles', vol. xv.

that the general course was forward in the dorsal vessel, downward in the hearts, and backward in the ventral vessel behind the hearts, but considered that the flow was forward in the ventral vessel anterior to the hearts. The lateral vessels are regarded as specializations of an intestino-tegumentary system present in each segment in the intestinal region—the flow in these lateral vessels was in a forward direction.

Bourne's scheme of circulation allows a complete intestinal circulation in each segment, blood leaving the dorsal vessel by dorso-tegumentary vessels to the integument, and passing thence to the gut by intestino-tegumentaries and back to the dorsal vessel by the dorso-intestinals. The ventral vessel supplied blood to the integument and to the intestine by ventro-tegumentary and ventro-intestinal vessels, but received no blood posterior to the heart.

In *Drawidia* (*Moniligaster*) *grandis*, Bourne (8) described latero-intestinal hearts arising from the dorsal vessel and from transverse vessels communicating with the lateral vessels, there being no supra-intestinal in this species. The circulation is essentially similar to that of *Megascolex caeruleus* (7) save that there are no intestino-tegumentary vessels and therefore no partially complete segmental circulation.

Harrington (9) studied the vascular system and circulation in *Lumbricus*. His scheme of circulation is based entirely on the direct observation of the pulsations seen in the vessels of small specimens under a dissecting lens. Harrington's conception of the circulation, which differs widely from that of Bourne and which allows a complete segmental circulation, has been superseded by the work of Johnstone and Johnstone.

Johnstone and Johnstone (10) give a careful account of the circulation of *Lumbricus*. Their methods are essentially similar to those used by Bourne. They believe that Harrington's errors can be attributed to his method of observation, since localized contractions would produce momentary reversals of flow in the blood-vessels and might be mistaken for pulsations. Their scheme agrees with Bourne's in most essentials with the exception of the course in the dorso-tegumentary vessels. These vessels, they state, return blood to the dorsal vessel from the



integument. Segmental circulation is impossible since the dorsal vessel collects blood from the organs, carries it forward to the cephalized region, where it is transferred by the hearts to the ventral vessel, and thence back to the body and gut.

Combault (reported by Stephenson (11)) considers that the flow is backward throughout the ventral vessel. He constricted the ventral vessel anterior to the hearts and found that posteriorly to the constriction it filled as the hearts contracted and emptied between contractions. He believed that this momentary reversal could be explained as a mechanism for regulating pressure in the ventral vessel and apparently ensuring an even flow of blood.

Bahl (2) gives a careful description of the blood-vessels and the course of the circulation in *Pheretima*. The general scheme of circulation is similar to that in *Lumbricus* as described by Johnstone and Johnstone. Posteriorly to the hearts no blood leaves the dorsal vessel, anteriorly to the heart all vessels associated with the dorsal vessel carry blood away from it. Ventro-tegmentaries and ventro-intestinals arise from the ventral vessel in every segment, and carry blood to the integument and the gut. The flow in the anterior portion of the ventral vessel is forward. Blood is collected from the anterior region by vessels communicating with the lateral vessel in which the flow is backward.

#### METHODS EMPLOYED FOR THE STUDY OF THE CIRCULATION

The largest specimens obtainable of *O. thomasi* were chosen. After narcotization, and in many cases a subsequent hypodermic injection of strophanthin, these were dissected from the dorsal, lateral, or ventral surface under physiological salt solution, and studied by means of a low-power binocular dissecting microscope. Strophanthin has the effect of increasing the blood-pressure and making the course of circulation easier to follow, and of causing the vessels to be dilated and so rendering small vessels easier to detect.

The course taken by the blood in the various vessels was determined by the following methods:

- (1) The direction of waves of contraction was noted. This

method was found to be applicable only to the pulsatile vessels, i.e. the dorsal vessels and hearts.

(2) The action of the valves was studied where they were present, and the course that the blood must take to pass them ascertained.

(3) The vessels were closed by means of clips made from fine spring wire. Increase of pressure on one side of the clip was shown by dilatation of the vessel, and decrease of pressure by partial draining of the blood.

(4) The vessels were cut or broken. More profuse bleeding from one end than from the other gave evidence of greater pressure on that side of the cut.

(5) An attempt was made to introduce traces of pigment to the vessels and to watch its subsequent distribution. A syringe fitted with a fine glass capillary-cannula was used; but, as the force required to overcome the capillarity becomes very great as the diameter of the tube is decreased, it was not found possible to use tubes fine enough to enter any but the dorsal vessel. As pigments indian ink and methyl violet were employed, the former proving less satisfactory as it tended to deposit solid particles and block the vessel.

#### DESCRIPTION OF THE BLOOD-VESSELS.

As in all earthworms there is a considerable difference between the blood-vessels in the cephalized and intestinal regions. In the intestinal region the system is relatively simple and, as it is repeated metamerically, may be considered to represent the typical arrangement of the blood-vessels. It is convenient to describe the vessels in this region first, as both Harrington and Bahl have done, and then to pass on to the more modified anterior region.

#### Segments Posterior to Segment 20.

##### Peri-intestinal Plexus.

This plexus consists of a network of blood-channels lying between the epithelial and muscular layers of the gut-wall. The vessels are so large and the meshes so small as to form an almost complete sinus. In the mid-dorsal and mid-ventral lines

there are in the intestinal wall typhlosolar and sub-intestinal tracts. These tracts have no definite walls, but are rather longitudinal dilatations of the blood sinus, with which they communicate freely. Connecting the typhlosolar and sub-intestinal tracts are two pairs of circular tracts which encircle the gut in the anterior and posterior third of each segment.

These circular tracts are also mere specializations of the sinus.

### Dorsal Vessels (*D.V.*).

The dorsal vessel in *O. thomasi* is represented by a pair of tubes running parallel to each other on either side of the mid-dorsal line. In the intestinal region the vessels lie close to the gut, and are covered by chloragogen cells continuous with those surrounding the tract.

Contrary to Beddard (4), who described the dorsal vessel as being 'completely' double in *O. thomasi*, I find that from the posterior end to the region of the gizzard the two tubes are segmentally connected by a definite transverse vessel. Each of the two dorsal vessels is slightly constricted as it passes through the septum, and at the constriction bears a pair of valves which open forwards, the nature of which will be described later. The connecting vessel, which is just anterior to the valves, is not a mere junction produced by the fusion of the two tubes but a short vessel of uniform diameter comparable with the rung of a ladder (fig. 5, Pl. 8). The relation of the connecting vessel may be shown beyond doubt by cutting one of the two dorsal vessels and emptying the blood from the other of the same segment by pressing it back across the connective; the back pressure is sufficient to close the valves at the septal constriction, rendering it impossible to force the blood into a posterior segment.

The dorsal vessels are rhythmically contractile and may be considered to consist of a series of segmental muscular chambers. Each chamber has a slightly conical appearance, being dilated posteriorly, and is marked off from those anterior and posterior to it by the septal constrictions and their valves. Into each chamber open two short dorso-intestinal vessels (fig. 2, Pl. 8, *D. int.*) which join the circular tracts of the intestinal plexus at their union with the typhlosolar tract. In addition, at the

anterior end of each segment just behind the septal constriction, each chamber receives a dorso-tegumentary vessel (*D.teg.*). The openings of the dorso-intestinals and dorso-tegumentaries are guarded by valves which project into the dorsal vessel directing the blood into it. At the posterior end of the worm each of the two dorsal vessels arises in the last segment by the union of capillaries from the gut and from the integument, which may be considered to represent the dorso-intestinal and dorso-tegumentaries of this segment.

#### Ventral Vessel (*V.V.*).

The ventral vessel extends from the anterior to the posterior end of the body lying in the mid-ventral line suspended by a mesentery from the gut. It is single throughout its length and is of uniform diameter.

In the intestinal region it gives off a pair of ventro-tegumentary and a single ventro-intestinal vessel in each segment. The ventro-tegumentaries leave the ventral-vessel just anteriorly to the septal wall and run for a part of their course on the anterior face of the septum. The ventro-intestinals arise from the vessel in the middle of each segment and run upward in the suspending mesentery to join the sub-intestinal tract of the peri-intestinal plexus. At the posterior end of the body the ventral vessel divides into branches distributed to the gut and integument.

There are no valves at any point in the course of the ventral vessel, which is non-contractile.

There is no sub-neural vessel in *O. thomasi* or other Acanthodrilid.

#### Tegumentary Vessels (figs. 2, Pl. 8, and 7, Pl. 9).

The dorso-tegumentary vessels (*D.teg.*) are paired and lie against the posterior face of the septum in each segment. They are formed by tributaries from the body-wall and nephridia and enter one or other of the dorsal vessels just posteriorly to the septal constriction.

The ventro-tegumentary vessels (*V.teg.*) are paired vessels leaving the ventral vessel just anteriorly to the septum in each segment. After running on the anterior face of the septum for a short distance they pierce it and enter the next posterior

segment. Here they ascend the septum circularly on its posterior face, and give off branches parallel to those which give rise to the dorso-tegmentaries.

There is no intestino-tegmentary system in this region.

### Vascular System in the Anterior Twenty Segments.

#### Alimentary Plexus and Associated Vessels.

The alimentary plexus consisting of blood-channels lying between the epithelial and muscular layers of the gut arises in the seventh segment, i.e. directly behind the gizzard. In segments 7 and 8 the plexus is represented by blood-channels lying in longitudinal folds of the epithelium. Posteriorly, the channels form a closer network approximating to a sinus which surrounds the epithelium and is directly continuous with the sinus of the intestinal region. In segments 8-13 there is in addition a more external network of capillaries lying beneath the peritoneum but outside the muscular layer. These capillaries communicate both with the internal plexus and with the lateral oesophageal and supra-intestinal vessels.

The supra-intestinal vessel (*S.I.V.*) lies on the dorsal surface of the oesophagus in segments 7 to 17 and communicates in many places with the internal plexus of which it may be regarded to be a specialization. Posteriorly, it comes to an end in the sinus between the oesophageal gland in segment 17. Anteriorly, its branches give rise to capillaries distributed over the gizzard. The vessel is single but bifurcates after passing through the posterior septum of each segment, the two branches coalescing towards the middle of the segment. The loops thus formed are dilated in segments 10-13 and receive circular vessels from the lateral oesophageal vessels. Coincident with the union of these vessels is the point of origin of the intestinal portion of the latero-intestinal hearts (figs. 3, 6, Pl. 8, and 9, Pl. 9).

#### Dorsal Vessel (*D.V.*).

Anteriorly to the large intestine the two dorsal vessels leave the surface of the tract though they are still bound to it by folds

of the peritoneum and lie higher in the body-cavity. The double nature of the vessel is retained up to segment 6, the two tubes no longer remaining parallel but curving away from one another between the septa. In segment 6 slightly anteriorly to the connecting vessel the two tubes coalesce, then separate, then coalesce again to form a single vessel which continues forward to the fourth segment (fig. 4, Pl. 8). In segment 4 over the pharyngeal mass it divides into four pairs of vessels. These are:

(a) posteriorly, a pair of commissurals passing down through the tufted pepto-nephridia to join the ventral vessels (figs. 1 and 4 A, Pl. 8);

(b) two pairs which are distributed over the pharynx, one transversely, the other obliquely forward (figs. 1 and 4 B, Pl. 8); and

(c) most anteriorly, a pair which, after giving rise to small branches to the integument, continue forwards obliquely dorso-laterally to the cerebral commissure, beneath which they divide, the largest branch of each joining a corresponding branch from the ventral vessel, the smaller branches being distributed over the wall of the buccal cavity (figs. 1 and 4 c, Pl. 8).

In each of segments 14-20 the dorsal vessels receive a pair of dorso-intestinal vessels and in segments 18-20 in addition a pair of dorso-tegumentary vessels. These vessels are similar to those in the intestinal region.

There are four pairs of latero-intestinal hearts in segments 10-13, two pairs of lateral hearts in segments 8 and 9, and a pair of commissurals in each segment anterior to this. These last are connected with the dorsal vessel, but the openings to the dorsal vessel bear no valves.

### Ventral Vessel.

The ventral vessel is continued forward to the second segment, where it bifurcates one branch going to each side of the buccal cavity, and after giving rise to small vessels to the integument, to the ventral surface of the pharynx, and to the walls of the buccal cavity, joins the branch (c) from the dorsal vessel beneath the circum-oesophageal commissure.

In segments 14-20 ventro-tegumentary and ventro-intestinal

vessels similar to those in the intestinal region leave the ventral vessel. Anteriorly to segment 14 it receives a pair of hearts or commissurals in each segment, but no vessels leave it before it bifurcates.

#### Lateral-oesophageal Vessels (figs. 1, 3, and 4, Pl. 8).

These are a pair of longitudinal vessels situated ventrolaterally to the oesophagus in segments 4-13. In segments 8-13 they are attached to the oesophagus and communicate with the peri-oesophageal plexus through numerous capillaries. In segments anterior to this they become free from the gut and lie in the body-cavity, being the most prominent vessels of this region.

In the segments containing the latero-intestinal hearts (segments 10-13) the lateral-oesophageal vessels communicate with the supra-intestinal by circular vessels in the wall of the oesophagus (fig. 3, Pl. 8), and may be considered to end posteriorly by joining the supra-intestinal by means of these vessels. The lateral-oesophageal vessels cease in segment 4 where they turn sharply upward and join to form a loop over the crop. On either side this loop receives five vessels which arise on the walls of the pharynx and buccal cavity (fig. 1, Pl. 8).

In each segment the lateral oesophageal vessels receive intestino-tegumentary vessels, and may be considered to form a part of the intestine-tegumentary system, as Bourne (7) suggested, since they communicate both with the integument by way of the intestine-tegumentaries and with the peri-oesophageal plexus.

#### Hearts and Commissural Vessels (figs. 1, 3, 6, Pl. 8, figs. 8, 9, Pl. 9).

Posteriorly to segment 13 the dorsal and ventral vessels have no direct communication with one another. But in segment 13, and in each segment anterior to it, there is a pair of hearts or a pair of commissural vessels connecting them. These connecting vessels are of three types.

(a) The latero-intestinal hearts (*L.I.H.*) in segments 10-13 communicate dorsally with both dorsal and supra-intestinal vessels and ventrally with the ventral vessel. Each heart con-

sists of three contractile pear-shaped chambers and a small ventral spherical bulb, arranged in series (fig. 9, Pl. 9). The dorsal chamber, which is much larger than the others, is connected to the dorsal and supra-intestinal vessels, the openings of which are guarded by valves opening towards the heart (fig. 10, Pl. 9). The constriction between the lowest chamber and the bulb is guarded by a collar valve which directs the blood into the ventral vessel with which the bulb communicates (figs. 3, 6, Pl. 8, figs. 9, 10, Pl. 9).

(b) The lateral hearts (*L.H.*) in segments 8 and 9 communicate with dorsal and ventral vessels but not with the supra-intestinal vessels. They are in the form of a series of contractile bulbs (fig. 8, Pl. 9). Near the origin from the dorsal vessel there is a valve opening towards the heart, and a short distance from the ventral vessel there is a second valve opening ventrally, i.e. away from the heart. Between this valve and the ventral vessel there arises a ventro-tegumentary vessel.

(c) The commissural vessels, of which there are five pairs, are non-contractile loops joining the dorsal and ventral vessels. In segments 5, 6, and 7 the commissurals bear at about one-third of its length from the dorsal vessel small bulbs, containing valves opening ventrally, above which no vessels leave.

Below this valve the commissurals in segment 7 give rise to two tegumentary vessels (fig. 11, Pl. 9); in segments 5 and 6 the commissurals give rise to two vessels on each side, the dorsal pair to the gizzard, the ventral pair to the integument (fig. 1, Pl. 8). The commissurals in segment 4 have no bulb or valves—they give rise to one vessel arising about half-way down and going to the pepto-nephridia and to another supplying the integument (fig. 1).

The anterior branches (c) from the dorsal vessel, where it divides above the pharynx, together with the branches from the bifurcation of the ventral vessel must be considered to be the most anterior pair of commissurals, since they unite to form a loop round the gut beneath the oesophageal nerve commissure and give rise to vessels with a distribution similar to those from the posterior commissurals, i.e. to the gut (pharynx and buccal cavity) and to the integument.



### Tegumentary Vessels.

(a) *Intestino-tegumentary Vessels* (fig. 1, Pl. 8, *Int.teg.*).—In the cephalized region the body-wall is served by a series of vessels connected with the intestinal vessels and not with the dorsal vessels as is the case in the posterior region of the body. The posterior pair of this series arises on the body-wall in segment 18 and continues forward on its ventro-lateral surface to segment 13 or 14. Here the vessels pursue a transverse course on the anterior face of the septa to within a short distance of the gut, where they leave the septa and continue forward on the oesophageal wall to join the lateral oesophageal vessels in segment 13. The actual segment (i.e. 13 or 14) in which these vessels traverse the septa has been observed to vary in different specimens and even on either side of the same specimen. The remaining intestino-tegumentary vessels are segmentally arranged and each is confined to one segment.

(b) *Ventro-tegumentary Vessels* (*V.teg.*).—The ventro-tegumentary vessels in segment 9 and in the segments anterior to this do not rise from the ventral vessel but from the ventral portion of the lateral hearts or commissurals of these segments and each is confined to one segment.

### The Valves.

The presence of valves has been noted in the description of the dorsal vessels and of the hearts and commissurals.

The valves in *O. thomasi* are of two types: (a) double, formed by two separate and opposed masses of tissue, and (b) circular or collar valve.

Double Valves (fig. 11, Pl. 9) occur at the septal constrictions of the dorsal vessels and also at the entrances of the dorso-tegumentaries and of the dorso-intestinals into these vessels. They are in the form of pear-shaped lobes attached by their apices. When the valves are opened the lobes float freely in the blood-stream, and when closed they fold back and meet, completely filling the aperture.

Circular or Collar Valves (fig. 10, Pl. 9) occur in the hearts and commissurals. They consist of a flap of tissue encircling the vessel and attached by one edge to its inner wall,

the other edge being free. When the valves are opened the flaps hang freely in the blood-stream; when closed they form transverse partitions preventing the flow of blood.

The action of the valves can be studied in a recently killed worm. If pressure is applied to a vessel behind the valve it opens widely and the blood passes through. The slightest pressure applied to a vessel in front of a valve, however, causes the valve to close and prevents the passage of blood through the opening. There seems to be no need to suppose that the action of the valves is due to the muscular activity of the valves themselves as Vejdovsky supposed (Stephenson (11)), but rather that there is the 'flap action' assumed by Stirling (Stephenson (11)) which changes in blood-pressure are sufficient to explain.

#### THE COURSE TAKEN BY THE BLOOD.

All authorities are agreed that the blood flows forward in the dorsal vessel, downward in the hearts, and backward in the ventral vessel behind the hearts in all earthworms, and the simplest observation is sufficient to confirm these facts.

#### Intestinal Region.

If the dorso-tegmentaries are cut or broken bleeding occurs from the distal end and not from the proximal end of the cut. Further, the valves which guard the entrances of the dorso-tegmentaries into the dorsal vessel allow the passage of blood to, but not from, the dorsal vessel. It can therefore be definitely stated that the flow in the dorso-tegmentaries is into the dorsal vessel.

The dorso-intestinals when severed do not bleed from the proximal end. Further the valves guarding their openings into the dorsal vessels close so as to prevent the blood passing into the intestinal vessels.

Methyl violet introduced to the dorsal vessel could be traced forward for some segments, but did not enter the tegumentary or intestinal vessels.

Thus it is evident that in the intestinal region the dorsal vessels received blood from all vessels connected with them.

When the ventro-tegmentaries are severed they

bleed freely from the ventral attachments but not at all from tegumentary capillaries.

The ventro-intestinals when severed bleed slightly from the sub-intestinal end and profusely from that attached to the ventral vessel, indicating that the pressure in the ventral vessel is much higher than in the intestinal vessels, and that the flow is from ventral vessel to the sub-intestinal tract in the intestinal plexus.

The flow in the tracts of the intestinal plexus is difficult to determine since they contain large quantities of blood at fairly even pressure. When cut the tracts bleed freely from both ends and, since they are closely associated with the peri-intestinal plexus, clipping merely causes the blood to take an alternative route. However, since the blood enters the intestinal system under pressure from the ventral vessel, and is removed by the dorso-intestinals during diastole of the dorsal vessel, the flow must be in general from ventral to dorsal round the gut.

In the intestinal region, therefore, blood passes from ventral to dorsal vessel by either of two routes, (1) through the ventro-tegmentaries to the nephridia and body-wall, through capillary plexus in the skin for oxygenation and thence by dorso-tegmentaries to the dorsal vessels, or (2) through ventro-intestinals to the gut-wall and thence by dorso-intestinals to the dorsal vessels.

The ventral vessel and the vessels that arise from it are therefore anatomically arterial, supplying blood to the various organs; the dorsal vessels and vessels associated with them are anatomically venous, collecting blood and returning it to the hearts in the anterior cephalized region. It is to be noted that there can be no complete segmental circulation such as Bourne (7) or Harrington (9) suggests since blood does not pass from dorsal to ventral vessels posteriorly to the hearts.

### Cephalized Region.

In the cephalized region the arrangement of the vessels differs from that posterior to it, and there is a corresponding difference in the course taken by the blood.

Anteriorly to segment 14 all vessels communicating with the

dorsal vessels receive blood from them. This is evident from the reversed action of the valves in the hearts and commissurals. The anterior commissurals which bear no valves, when severed bleed from the dorsal portion indicating a flow of blood from the dorsal vessels. Anteriorly to segment 14 the ventral vessel communicates with no vessels save the hearts and commissurals.

The integument receives blood from the ventro-tegmentaries which posteriorly to segment 14 arise from the ventral vessel, anteriorly to segment 8 from the lateral hearts and commissurals, and between segments 8 and 14, from branches from these vessels continued forward or backward on the body-wall. Blood is collected from the integument by the dorso-tegmentaries posterior to segment 18 and anteriorly to this by the intestino-tegmentaries which return it to the lateral-oesophageal vessel. The oesophagus receives blood from the ventro-tegmentary posteriorly to segment 14 and anteriorly to this from the lateral vessel. The gizzard is supplied by vessels from the commissurals in segments 5 and 6, the pharynx from branches of the dorsal vessel and also with the walls of the buccal cavity from the anterior commissural. Blood is collected from the oesophagus and gizzard by the supra-intestinal vessel, and from the pharynx and buccal cavity by the anterior branches of the lateral oesophageal vessels.

The lateral oesophageal vessels severed in segment 6 bleed from both ends, but more profusely from the anterior portion. Flow in the lateral vessel is therefore backward to the oesophagus and circular vessels.

Flow in the supra-intestinal is either forward or backward to the hearts in the region of which there is a reduction of pressure at each diastole.

Flow in the commissurals is towards the ventro-tegmentary vessels. In the portion which lies between these and the ventral vessel it is more difficult to determine. When cut this portion usually bled freely from both ends; in three specimens, however, the bleeding was distinctly more profuse from the ventral portion, which indicated that the flow was towards the ventro-tegmentary from the ventral vessel.

The ventral vessel when severed some distance anteriorly to

the hearts bled freely from both ends indicating a fairly even pressure within the vessel. When clipped at about the same point, no dilatation could be detected. When the ventral vessel was clipped nearer the heart it became dilated posteriorly at each contraction of the heart, the dilatation becoming reduced between contractions. The blood did not drain from the vessel between contractions of the heart as Combault stated to be the case.

Blood enters the ventral vessel from the heart at each contraction, and flows forward in front of and backward behind the hearts. Of the blood entering the anterior portion of the ventral vessel a part appears to be tidal and flows back past the hearts as the pressure is reduced between contractions, and a part continues forward and leaves the vessel through the ventral portion of the commissurals. As further evidence of the forward flow in the ventral vessel it may be noted that the paired lateral vessels are of much greater capacity than either the dorsal or ventral vessels of this region. It is evident, therefore, that the lateral oesophageal vessels are returning much more blood from the anterior region than the dorsal vessel alone could supply, and the extra blood can come only from the ventral vessel.

Fig. 12, Pl. 9, gives a diagrammatic representation of the course of the circulation in both intestinal and cephalized regions.

#### COMPARISON WITH OTHER TYPES.

The vascular system which has been described agrees in all essential respects in regard to the distribution of similar vessels and general scheme of circulation with the accounts of recent authors.

It agrees with *Lumbricus* in that there is no secondary system of vessels supplying the intestine either directly from the integument as occurs in *Megascolex*, or indirectly from the integument through septo-intestinals from sub-neural-dorsal commissurals as is the case in *Pheretima*.

In the cephalized region no vessels supply blood directly from the longitudinal trunks to the integument in the segments containing the hearts. In this respect *Lumbricus* and *Megascolex* are similar whilst *Pheretima* is different.

The absence of vessels to the integument arising directly from the ventral vessel in the region anterior to the heart is not paralleled in other types. However, if the commissurals were incomplete and the branch from the dorsal vessels supplied the gut, and the ventral vessel the ventro-tegmentaries, a system would be obtained almost exactly similar to that in *Pheretima*.

The circular vessels which put the lateral oesophageal vessels in communication with the supra-intestinal at the origin of the later-intestinal hearts from the latter, form an intermediate condition between the relations of the lateral oesophageal vessels in *Pheretima* where they communicate with the supra-intestinal and thence with the hearts, and in *Darwidia* where the circular vessels communicate directly with the hearts.

#### SUMMARY.

1. Posteriorly to the gizzard the dorsal vessel of *O. thomasi* is double, consisting of two tubes joined by a short connecting vessel anterior to the septum in each segment.

2. There are six pairs of strongly contractile hearts in segments 8-13. The latero-intestinal hearts in segments 10-13 receive blood from the dorsal vessel and from a common trunk from the supra-intestinal and latero-oesophageal vessels, and supply blood to the ventral vessel. The lateral hearts receive blood from the dorsal vessel and supply ventro-tegmentary and ventral vessels. For complete circulation all blood must pass through the hearts.

3. The ventral vessel, which is not contractile, receives blood from the hearts; posteriorly to the hearts the flow is backward in the ventral vessel and outward via ventro-tegmentaries and ventro-intestinals to the body and gut; anteriorly to the hearts the flow is forward, the blood leaving the ventral vessel by the commissures from which the ventro-tegmentaries of the anterior region arise. The ventral vessel is the main arterial trunk.

4. Posteriorly to the hearts the dorsal vessel collects blood from the body and gut by dorso-tegmentaries and dorso-intestinals. The flow in the dorsal vessel, which is contractile, is forward.

Blood leaves the dorsal vessel through small vessels to the latero-intestinal hearts, and larger vessels to the lateral hearts. Anteriorly to the hearts, the commissures and all vessels connected with the dorsal vessel receive blood from it. Thus, posteriorly to the hearts the dorsal vessel is venous, and anteriorly to the hearts arterial, in character.

5. Blood from the anterior region is returned by paired latero-oesophageal vessels to the supra-intestinal vessel and to the latero-intestinal hearts. The latero-oesophageal vessels and the supra-intestinal vessels are the main venous trunks in the anterior region.

6. There is no sub-neural vessel.

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## EXPLANATION OF PLATES 8 AND 9.

## LETTERING.

Hearts and commissures—untouched vessels.

Arterial vessels, i.e. vessels carrying blood from hearts and supplying body and gut—banded vessels.

Venous vessels, i.e. vessels returning blood to hearts:

Dorsal vessel—cross hatched.

Other vessels—black.

*a.*, *b.*, and *c.*, anterior branches of dorsal vessel; *Buc.*, Buccal cavity; *com.*, commissural vessel; *con.*, vessel connecting dorsal vessels; *Cr.*, Crop; *ct.*, circular tract; *D.int.*, Dorso-intestinal; *D.teg.*, Dorso-tegumentary; *D.V.*, Dorsal vessel; *Giz.*, Gizzard; *Int.*, Intestine; *int.teg.*, intestino-tegumentary; *i.p.*, internal plexus; *L.H.*, Lateral heart; *L.I.H.*, Latero-intestinal heart; *L.V.*, Latero-oesophageal vessel; *N.*, Pepto-nephridia; *o.c.*, circum-oesophageal commissure; *Oes.*, oesophagus; *Oes.Gl.*, Oesophageal gland; *S.I.V.*, supra-intestinal vessel; *Sept.*, septum; *Sub.int.*, Sub-intestinal; *t.t.*, typhlosolar tract; *V.int.*, Ventro intestinal; *V.teg.*, Ventro-tegumentary; *V.V.*, Ventral vessel.

## PLATE 8.

Fig. 1.—Lateral view of gut and blood-vessels in segments 1–20. The septa are indicated by the short vertical lines above and the segments by Roman numerals.

Fig. 2.—Lateral view of a segment in the intestinal region with body-wall cut away above and below leaving the septa free.

Fig. 3.—Lateral view of segment 12 to show the relations of the latero-intestinal parts to the vessels of that segment.

Fig. 4.—Dorsal view of segments 1–8.

Fig. 5.—Dorsal view of dorsal vessel in intestinal region.

Fig. 6.—Dorsal view of segment 12 to show relations of dorsal and supra-intestinal vessels and the latero-intestinal hearts.

## PLATE 9.

Fig. 7.—Composite transverse section in the intestinal region:

To the left: through a circular tract showing septum and tegumentary vessels.

To the right: septum incomplete ventrally to show the origin of the ventro-tegumentary vessel in the preceding segment.

Fig. 8.—Composite transverse section through segments 7 (right) and 8 (left), to show in one the commissural and the other the lateral hearts. The origin of the tegumentary vessels is shown.

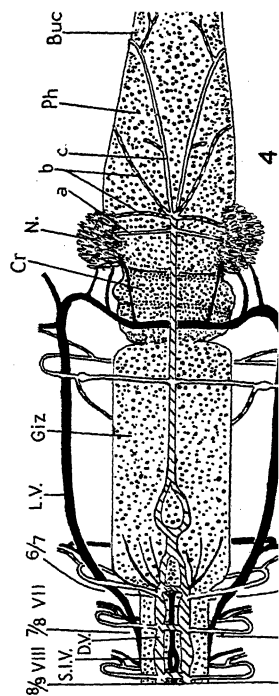
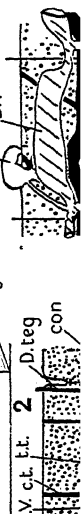
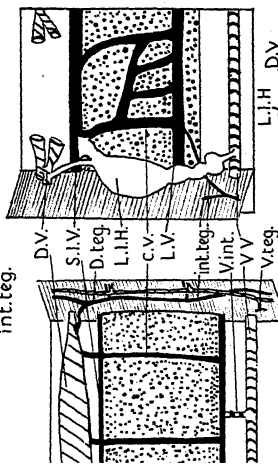
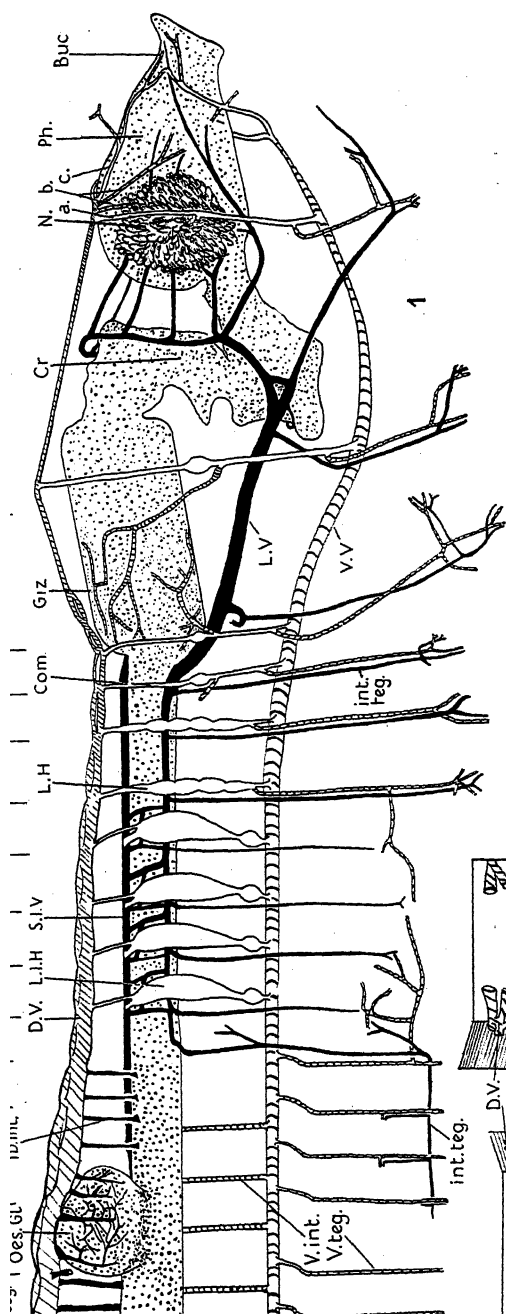
Fig. 9.—Transverse section through segment 12 showing latero-intestinal hearts, origin of tegumentary vessels, circular tracts, and inner plexus.



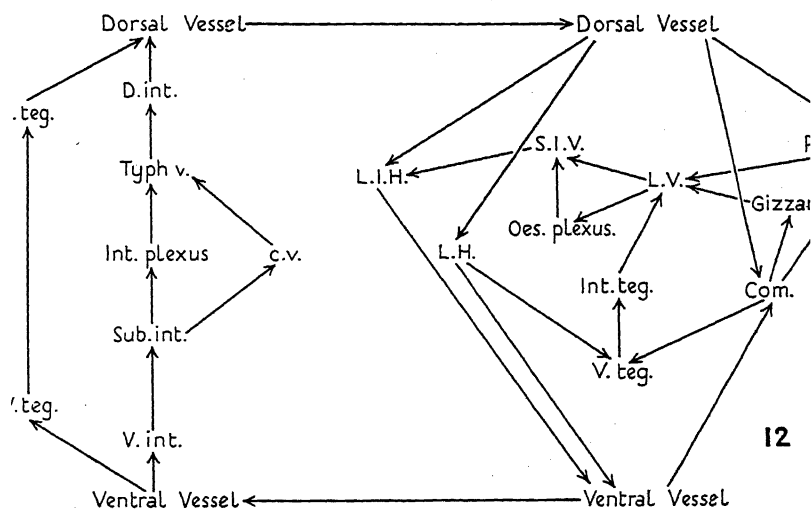
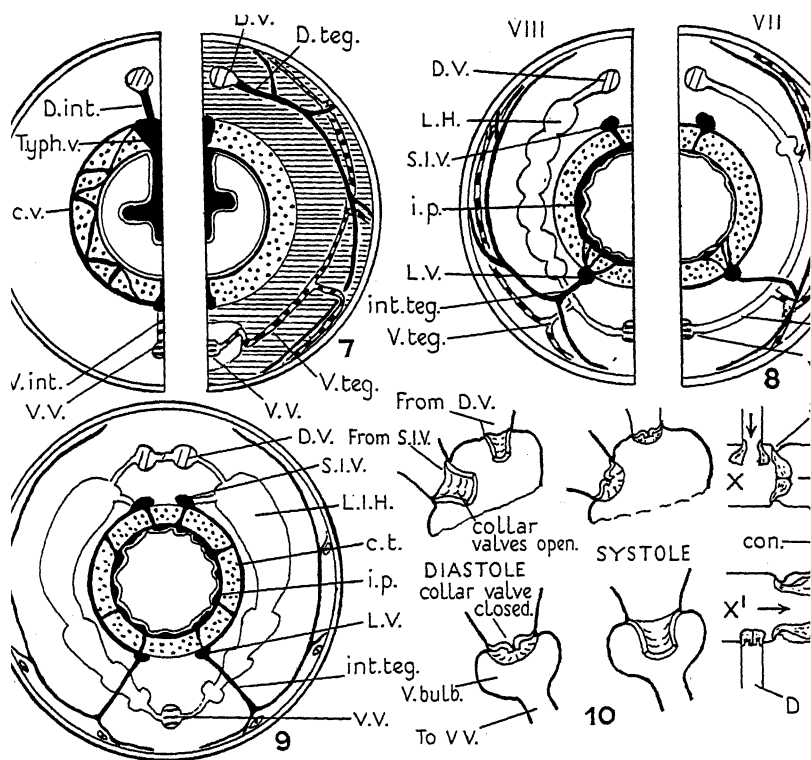
Fig. 10.—Diagram to show the action of the collar valves in the latero-intestinal hearts in diastole (left) and systole (right).

Fig. 11.—Diagram of the double valve in the region of a septal constriction of the dorsal vessels. Chambers X and Y' diastole with valves guarding the openings of *D.teg.* and *D.int.* vessels and posterior septal constriction open and valves guarding anterior septal constriction open. Chambers X' and Y systole.

Fig. 12.—Tabular diagram to illustrate the course taken by the blood in the intestinal and cephalized regions.









# On the Formation of the Ground Substance of Loose Connective Tissue

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With Plate 10.

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THE formation of the ground substance of connective tissue has for long been one of the most controversial problems of histology. In spite of the diversity of the material studied, and of the methods of investigation used, there exists on this problem as yet no generally accepted view. Thus, we have at present several theories treating differently of the process of the formation of the ground substance and of its fibres.

Ever since the theory according to which the collagen fibres<sup>1</sup> are considered as proceeding from an immediate transformation of cytoplasmic structures, such as the fillar mass and the chondriosomes (Flemming, Reinke, Spuler, Meves) has been discarded, the said problem has been reduced to the study of the formation of the amorphous substance in which and from which the fibres originate. Since the amorphous substance was recognized to be the product of cell activity its formation might be supposed to occur either by way of secretion or by transformation of the protoplasm.

On the theory that the amorphous substance arises from cells by secretion (Merkel, v. Ebner, Petersen, Plenk, and others) the origin of fibres from this substance is considered certainly not as a phenomenon occasioned by endogenous formational potentialities of the protoplasm, but as a result of mechanical relations and forces acting upon the amorphous substance produced by the cells; in this lifeless amorphous substance the

<sup>1</sup> The problems concerning the elastic fibres are not included in the present study, which deals only with collagen fibres.

fibres are formed under the influence of 'pressure and tension' (Zug und Druckspannung, Roux). From the point of view of the authors adhering to the theory of the transformation of protoplasm (Hansen, Studnička, Mall, Laguesse, Schaffer, Zawarzin, Hueck, Wassermann, and others) the process of formation of the ground substance takes another course. According to them, the fibres do not originate directly from intracellular formations, nor may they be considered as a simple product of cellular secretion. In the primary stages of development the fibres originate from the protoplasm lying in the periphery of the cell, from the so-called ectoplasm. What remains of this ectoplasm after fibre formation represents the amorphous 'cementing' substance (Kittsubstanz) that lies between the fibres. From this point of view the ground substance (metaplasm), fibrillar as well as amorphous, while losing in the course of the next developmental stages its direct connexion with the cells that have changed into pure endoplasmic elements, retains nevertheless a certain minimum of vital properties; thus it is capable of further quantitative accretion and production of fibres independently of the cells. In other words, it is capable of so-called 'formative activity'. Thus the ectoplasmic theory admits of two ways of formation of the ground substance—in early stages it is produced at the expense of the ectoplasm, while in subsequent stages it is increased by its own metabolism.

Besides the theories cited there exists another theory (Nageotte) which generally denies the existence of amorphous ground substance of a particular character. According to this theory the fibres are contained directly in the colloidal interstitial liquid, from which they originate, in the case of fibrin, under the influence of a certain hypothetical ferment secreted by the cells; at the same time it is to be understood that the fibres are not possessed of any vital property. This theory is particularly supported by the authors who have studied the formation of fibres in tissue cultures. Thus, for instance, Baitsell came to observe in cultures the formation of fibres from nutritive plasm and fibrin. In respect of tissue cultures Baitsell's opinions have been refuted by Chlopin. To begin with, the latter author has observed the formation of fibres in areas

of the explant containing no fibrin. Considering the experimental conditions used by Chlopin, it would further have been necessary to admit that human mesenchymal cells evoke the formation of fibres in the plasm of the chicken embryo, i.e. in a foreign plasm—which from the point of view of biology seems hardly possible. It would be more correct to admit, as Chlopin remarks, that the fibres originate in a substance that in some way is produced by the cells of the tissue culture. However, for investigators who consider the formation of fibres to be a solely physico-chemical process, these considerations are scarcely convincing.

A comparison of the above-mentioned theories concerning the formation of the ground substance leads to the conclusion that, at least in respect of the early stages of development, only the theory of the transformation of protoplasm, i.e. the ectoplasmic theory, rests on proofs based on direct observations (though not all the investigators consider these proofs as equally convincing). The secretory and fermentative theories are entirely bereft of such proofs; they are solely based on hypotheses and exclusions of other possibilities owing to the lack of facts.

Concerning the secretory theory it is necessary to point out that the authors supporting it never speak directly of secretion, but describe the process by means of which, according to them, the ground substance is produced as a 'segregation' (Ausscheidung, Abscheidung, Absonderung). They do not speak of secretion because, as a matter of fact, judging by the descriptions given, they never observed secretion; they just saw that a certain substance in which the fibres arise appears about the cells, that it seems to be produced, 'segregated', by them. As the supporters of the 'segregation' theory have not discovered either secretion or an immediate transformation of cellular protoplasm into the said substance (which the supporters of the theory of the transformation of protoplasm designate as ectoplasm), and as they wish to state that this substance is produced by certain cells,<sup>1</sup> they prefer to use a neutral expression, 'segregation', that, properly speaking, explains nothing. In this

<sup>1</sup> The word 'cell' is here equivalent to the endoplasm of the 'ectoplasmists'.



respect certain authors (Petersen, Plenk, and before them v. Ebner) entirely change the point under discussion. They say that, as a matter of fact, there is no difference whatever between transformation of the protoplasm and secretion, and that the discussion is one merely of words. Thus, according to Petersen: "Jede Secretion kann eine Umwandlung genannt werden, denn die Materie, die später als Sekret erkennbar wird, steckt vorher in der Zelle, ist ein Teil des Protoplasten. Die geringere oder grössere Geschwindigkeit in der Ergänzung des verbrauchten Materials ist es, die den Vorgang unter Umständen als Umwandlung von Zellteilen erscheinen lässt, nämlich ob der Protoplast daher an Grösse abnimmt oder nicht. Die lebende Substanz vollzieht in allen Fällen eine Synthese, leitet sie wenigstens ein, und von einer Umwandlung sollte man nur dann reden, wenn vom Protoplasten nichts, d.h. kein regenerationsfähiges Gebilde übrigbleibt." As a matter of fact, even leaving out of consideration that the product of secretion is accepted as dead substance, while transformed substance is reckoned to retain to a certain degree the character of a live substance—even in this case, the discussion is not solely one of words. Such would indeed be the case if the physiological part of the process alone were discussed, as from this point of view it is really hardly possible to distinguish between secretion and transformation of the protoplasm; or if it were previously established and known that the ground substance is produced by certain cells; or, finally, if there did not exist a theory, according to which the cells really secrete, not the ground substance, but the ferment under whose action collagen fibres arise from the interstitial liquid. As a matter of fact the circumstance that ground substance is produced, 'segregated', by cells and withal by one kind or other, remains yet to be proved; and this of course requires an indication of the mode, of the form of this 'segregation', because the expression 'segregation' (Abscheidung) without this has no meaning whatever. Three forms of secretion—the mero-, apo-, and holocrine—are known to us. If the adherents of the 'segregational' theory were able on the strength of their investigations to affirm that the process of 'segregation' takes the form of an apo- or holocrine secretion,

their observations would to a certain degree coincide with the data of the ectoplasm theory. But they are not able to make such an affirmation, because according to their descriptions and illustrations the cellular protoplasm and the ground substance are always distinctly demarcated from each other. As to the merocrine secretion, this form has been observed, to my knowledge, only by Renaut and Dubreuil.<sup>1</sup> However, Dubreuil, for instance, abstains from giving an opinion as to whether the product of this secretion is represented exactly by the ground substance itself or by some ferment acting on the interstitial liquid, and, consequently, he gives no definite answer to the question concerning the formation of ground substance. Thus the discussion on 'secretion or transformation of cellular protoplasm' is by no means a simple discussion of words—it is concerned with the way in which the cells take part in the formation of the ground substance, and also further with the question what cells exactly participate in its production.<sup>2</sup>

<sup>1</sup> According to v. Korff odontoblasts and osteoblasts possess secretory granules. The author supposes that these cells produce by way of secretion (merocrine) a special matter that gives to the ground substance the capacity of retaining calcium salts.

<sup>2</sup> In a recently published study by Snessarew the ectoplasmic theory is rejected because the author could not distinguish on his sections (silvered according to the method of Bielschowsky) ectoplasmic and endoplasmic constituents in the protoplasm. At the same time he gives the following opinion on the formation of the ground substance, which opinion is difficult to understand, and it is not quite clear on what it is based. According to Snessarew the argyrophil fibres appear on the surface of the cells as a result of the precipitation of a fibrogenous colloid substance, which is non-protoplasmic, but changeable into protoplasm. The fibrogenous substance may be located in the cytoplasm, as well as in the interstitial liquid on its surface. The proto-paraplastic substance is the prototype of the ground substance, in some of its properties it is similar to protoplasm, but yet it is not protoplasm. It is also active and stands in immediate connexion with the protoplasm. It is neither a product of secretion, nor represents a product of the transformation of protoplasm. When the fibres are being formed their germs appear in the beginning as granules, rods, or small masses of matter, and together with these ring-like formations can be seen partly resembling Bowen's osmiophile plates, only being located outside the cells. The author (p. 719) 'is inclined to ascribe to these formations the role of "organizers" of the cementing substance'.

As regards the formations of the fibres, the difference of opinion pertains principally to the question whether they are of intra-, epi-, or extracellular provenance. These terms are clearly dependent on the point of view from which the cell is considered, and different authors according as to whether they recognize or not the existence of ectoplasm, whether they consider the ectoplasm as being a part of the cell, &c., do not always understand these terms alike. At all events, the diversity of opinion as to where the fibres are formed is due not so much to facts as to their interpretation, which in turn depends on the point of view of each author as to the origin of the fibres and of the amorphous substratum producing the fibres. Generally speaking it must be said that the assertions concerning the place of formation of the fibres, while not helping essentially in the clearing up of the question as to their origin, ought to be considered rather as resulting from the author's opinions on the formation of ground substance, and can hardly serve as a basis for discussion.

Before we turn to the discussion of the formation of the ground substance of loose connective tissue, there is another general problem of importance concerning the formation of fibres to be mentioned, namely: whether the fibres in their development always go through the argyrophil, precollagen phase, or whether they may appear immediately as collagen fibres. The common opinion is (see, for instance, the study by Alfejew, 1926, p. 152, as well as Maximov's compendium, 1927, pp. 508-16) that these arise always as argyrophil bodies that only subsequently, in the course of their 'ripening', change into collagen fibres. But there also is another opinion. Thus Petersen (1924, p. 168) considers it to be impossible that every collagen fibre is preformed as an argyrophil fibre. Plenk supposes that probably in most cases the collagen fibres are formed without passing through the argyrophil phase; in other words, he considers with Petersen the collagen fibres to be as to their mode of formation of twofold origin.

Turning to the problems especially pertaining to the development of the loose connective tissue we must mention the corresponding data obtained by Studnička, who studied the

earliest stages of the formation of ground substance, for the most part on lower vertebrates as well as of Laguesse, who in his studies on mammals has confirmed in a general way the observations made by Studnička, and who together with the early stages has also investigated the subsequent developmental stages of loose connective tissue.

At the earliest stages of their development the embryonic layers prove to be connected to each other by protoplasmic bridges—plasmodesms, that as a whole Studnička designates as mesostrome (primary). The mesenchyme is formed in such a way that the nuclei of the mesoderm enter these bridges. Thus it comes to pass that the mesenchymal elements are from the very beginning interconnected with each other, forming a sort of network or sponge-like structure. Further, the elements of mesenchyme, while dividing, remain connected to each other not only by means of the larger processes but also by fine protoplasmic threads, the cytodemes. In stained preparations of fixed or supravital material these cytodemes differ from the protoplasm surrounding the nucleus, being homogeneous and showing a different staining reaction. Thus Studnička distinguishes already at this stage an endoplasm about the nucleus and an ectoplasm from which the cytodemes are made up. In the course of the further development of the mesenchyme, while the division of its elements continues, not only the processes connecting the mesenchyme cells with each other, but also the periphery of the cellular body becomes ectoplasmic. The ectoplasm belonging to each such cell is designated by Studnička 'autectoplasm'. If the material be suitably treated argyrophil fibres appear in the cytodesm as well as in the autectoplasm.

In the course of development of the loose connective tissue the star-like mesenchymal elements, as described by Laguesse, become flattened and at the same time the distance between them becomes narrower, while the mass of ectoplasm grows larger. The tissue acquires a lamellar structure, these lamellae being at first perforated, while later on they become continuous; they are connected to each other by obliquely directed bridges. The lamellae, between which lies interstitial liquid, contain together with the amorphous ectoplasmic substance also

argyrophil fibres that subsequently change into collagen fibres. On the other hand, the endoplasmic areas, that at first undergo reduction because it is from them that the ectoplasm arises, undergo regeneration, become more voluminous, and are distinctly delimited from the ectoplasm, lying on the surface of the lamella of ground substance, forming fresh endoplasmic processes there by means of which a connexion between the said areas is brought about. These very same flattened, anastomosing, purely endoplasmic areas represent the definite fibroblasts, the individualization of which from the ectoplasmic ground substance at the birth of the animal (rat, for instance, Laguesse, 1921, p. 226) comes to a close.

In what way does the subsequent increase of the ground substance and fibres in the course of the further development and growth of the animal take place, when the tissue contains only these fibroblasts bereft of ectoplasm? For, it must be held in mind that all that has been said up to the present on the formation of the ground substance pertains to developmental stages during which the cell elements (i.e. the nucleo-endoplasmic areas) still possess ectoplasm, representing the source of the material serving for the formation of the ground substance. When attempting to solve this question (very often, however, its discussion is omitted) the supporters of the ectoplasmic theory (Studnička, Hueck, Wasserman, and others) get away from facts and have recourse to supposition. In view of the impossibility of fixing upon such a source of material for the further neoformation of the ground substance as might lend itself to observation, they presume that the latter continues normally to increase independently of the cells.<sup>1</sup> This hypo-

<sup>1</sup> The supporters of the segregational theory do not need to have recourse to further hypothesis in order to explain the formation of the ground substance in the next developmental stages. From their point of view the ground substance in these stages is produced by the cells in the same way as in the early stages, namely by way of 'segregation'. Thus, for instance, Maximov (1927, p. 517) restricts himself to mentioning in small print that: 'Im erwachsenen Organismus ist überall im Bindegewebe durch die Anwesenheit von Fibrocyten oder undifferenzierten Mesenchymzellen potenziell die Möglichkeit der Neubildung von amorpher und fibrillärer Interzellularsubstanz gegeben.'

thesis is undoubtedly the most vulnerable point of the ectoplasmic theory.

Summing up, it should be pointed out that the facts given by Studnička and Laguesse pertaining to the initial stages of the formation of the ground substance as well as those on the development of loose connective tissue, in so far as they are not generally accepted, demand confirmation. As regards the subsequent stages, all the existing opinions being based but on hypothesis, the problem pertaining to them remains unsolved.

On the other hand, it must be mentioned that certain essential facts pertaining to the formation of the ground substance in the subsequent stages, that withal speak in favour of the ectoplasmic theory, were discovered by me in 1928. While investigating the cellular forms of the loose connective tissue I could ascertain that the fibroblasts in all post-embryonic stages are not purely endoplasmic elements as they are considered to be by Studnička, Laguesse, and other authors, but are built up of endo- as well as ectoplasm, i.e. they prove to be like the mesenchymal elements, but considerably more distinctly not mono-, but diplasmic cells. Further, these preparations led me to ascertain not only the presence of ectoplasm in fibroblast, but also that this amorphous transparent ectoplasm arises by way of direct transformation of the fine alveolar endoplasm with which it makes an indissoluble whole.<sup>1</sup> Further, I could

<sup>1</sup> Maximov (1927, p. 515) considered it doubtful whether the ectoplasm is also present in those cellular elements in which it has been described by Studnička and Laguesse. Bidermann considers one might speak of an ectoplasm of the connective tissue cells only if an exterior layer of protoplasm were found, analogous to that of the protozoan and forming an indissoluble whole with the endoplasm. Presumably of the same order of ideas is Petersen's denial of the presence of ectoplasm as described by Hansen in cartilage cells, basing his arguments upon the fact that when the cartilage cells shrivel during fixation, their endoplasm usually separates from the ectoplasm, while the latter remains in contact with the ground substance of the cartilage. Such being the case, Petersen considers the ectoplasm of the cartilage cells as a ground substance 'segregated' by the latter, since otherwise one would have to suppose that the semipermeable membrane lies between the endo- and the ectoplasm. However, the fact must be taken into consideration that even during typical plasmolysis in vegetable cells there takes place, as has been proved by Hecht, 'ein Zerreißen der

ascertain that the amorphous ectoplasm of fibroblasts, increasing continuously at the expense of the endoplasm, suffers at the same time uninterrupted consumption; a fact testified by the presence of holes of various sizes observed in it.

On the basis of these facts I considered myself entitled to affirm that, in the stages following the initial stages of development, the formation of the ground substance also occurs at the expense of the ectoplasm, though the latter is sharply contoured in its periphery and is clearly delimited from the ground substance.

It was necessary, however, to complete the data I had obtained by systematic investigations of the formation from the earliest stages of the ground substance of loose connective tissue, and next to confirm them by the data that might be discovered by investigating the formation of the ground substance of other categories of connective tissue.

As correctly pointed out by Laguesse, the development of mesenchyme into adult connective tissue may occur in different ways. Thus the loose connective tissue develops from mesenchyme after the 'lamellar mode' (mode lamelleuse), while, for instance, in bone, dentine, and cartilage the development takes place after the 'compact mode' (mode compacte). Undoubtedly, a clear idea of the process of the formation of ground substance may best be obtained by a comparative study of the kinds of connective tissue which develop either in one way or the other. Indeed, by such means facts are discovered that complete each other and by comparing which it becomes possible to elucidate from different points of view the problem in question and thus to come nearer to its correct solution.

As material for studies of the formation of the ground substance I made use of the subcutaneous loose connective tissue, that I had already previously studied, and of dentine, and bone as well.

I observed the early stages of development of mesenchyme on chicken embryos of 48-60 hours of incubation. As to the loose connective tissue, I studied the development of its ground  
bis dahin kontinuierlichen Protoplasamasse, eine Entrindung des Protoplasten'.

substance in different mammals (mouse, rabbit, cat), their embryos of different ages, as well as in new-born, young, adult, and aged individuals. In these investigations I made use of membrane preparations, made and stained after the method proposed by me (1932), and also of histological sections, made and treated in the usual way. The material used was fixed after Laguesse (1921, p. 175) and imbedded in paraffin.

On cross as well as tangential sections through the loose connective subcutaneous tissue I observed appearances analogous to those described and represented in his figures by Laguesse. This led me to ascertain that the loose connective tissue in all its developmental stages is in fact constructed of lamellae connected with each other by slanting bridges, the space between the lamellae, that in preparations remain entirely transparent, being evidently filled with interstitial liquid.<sup>1</sup> As to the fibroblasts, they show, though less clearly, the same diplasmic structure in paraffin sections (Text-fig. 1), as in the membranes, prepared according to my method<sup>2</sup> (Text-fig. 2).

<sup>1</sup> Petersen supposes the lamellar structure of the loose connective tissue to be an artefact resulting from fixation or some other cause (such as the introduction of liquid or of air), and that in reality the loose connective tissue consists of alternating layers of colloidal substance in the states of sol and gel.

<sup>2</sup> It is to be noted that Laguesse (for instance, in the new-born rat, p. 225, fig. 4), as well as v. Möllendorff and Pfuhl give in their illustrations the true contours and dimensions of fibroblasts. However, they did not succeed in bringing to light the diplasmic (endo-ectoplasmic) structure of these cells. In his later work on connective tissue Pfuhl, who in many respects has confirmed my observations, comes to distinguish a more dense, deeply staining 'centroplasm'.

Yet the membrane preparations gave occasion to certain doubts (see, for instance, Maximov, 1929), which at present are set at rest by the fact that I have been able to demonstrate the same relations in paraffin sections.

The method of preparation and treating of the membrane preparations I have proposed differs from the method used by v. Möllendorff as well as by Pfuhl in that I begin by fixing the material, and prepare the membrane only afterwards, while the authors named begin by preparing the membrane from fresh material, and fix it later. It is quite clear that my method in respect of the preservation of structural relations in the tissue under investigation, as in respect of the easiness of achieving the treatment of the



Here, both in membrane preparations as well as in sections, especially cross-sections, one may observe that the fibroblasts are disposed on the surface of the lamellae of which the loose connective tissue is composed. In both preparations, next to the fibroblasts lying in the form of bas-reliefs on the surface of the ground substance and closely cemented to it, there are often found loose fibroblasts. This loosening is just such an artefact as may be often observed in preparations owing to the tearing off of the elements of dentino- and osteoblastic plasmodium from the corresponding ground substance. As I had occasion to ascertain quite distinctly, in well-fixed preparations in my investigations on the formation of dentine (1924, 33) and of bone (1934), that the ectoplasm of the dentino- and osteoblastic

material, is more sure and leads to results of greater reliability. The advantages presented by a preliminary fixation of the material in the preparing of membranes is denoted also by Benninghoff. Of course with this method of treatment, as with any other, it is impossible to guarantee a complete preservation of the morphological relations of the living material. Thus it is possible, that during the distention of the membrane (it is understood that the membrane preparation must represent, as Pfuhl rightly says, the membrane as it exists in the loose connective tissue and not a so-called 'Zupfpräparat') some of the particularly fine ectoplasmic bridges between fibroblasts may be torn. But, on the other hand, it is quite impossible to admit a breaking up of all such connexions, if these really existed, and supposing that in the adult connective tissue all the fibroblasts are connected to each other forming a net. At least in the sections I make now, in which the tissue has not been subjected to distention, and in which the bridges might have been visible, they are nevertheless not observed. But even in the case of such a fibroblastic network (v. Möllendorff's 'Fibrocytennetz') existing in the adult loose connective tissue in some animals, the network would at all events not be such as it is described by Laguesse and v. Möllendorff, i.e. the fibroblasts in it would be connected to each other not endo-, but ectoplasmically. Studnička in his recent work (1933, p. 17) is of the same opinion.

It is to be noted that, even with certain practice in producing membrane preparations, entirely good preparations are not always obtained. Thus, for instance, the fine alveolar structure of the endoplasm of fibroblasts in my preparations, reproduced in my work of 1928 on figs. 9, 13, and 17, is considerably less clear than in the preparations reproduced in figs. 1, 2, and 6 of the same work, though the former as well as the latter figures express with all possible exactness the appearances observed as well as figures of the present work.

plasmodium may undergo a direct transition into the ground substance, there is no occasion to doubt that in the loose connective tissue also there takes place a similar direct transition of the fibroblast ectoplasm into the underlying ground substance. But if, as with dentine and bone, the transition may be displayed quite distinctly by a suitable treatment of the material, in the loose connective tissue the said transition is not displayed with the same clearness, as the fibroblasts are extremely flat cells and of scant thickness (the transition being discernible only in cross-sections). As regards the holes in the ectoplasm of fibroblasts I could now ascertain by careful examination that holes of that sort may frequently<sup>1</sup> be observed also in the ectoplasm of the dentino- and osteoblastic elements in the places of transition of this ectoplasm into corresponding ground substance. These holes in the ectoplasm must be considered as occasioned by the thinning of the ectoplasm, resulting in consequence of its changing into ground substance.

In general, when comparing the preparations pertaining to the early as well as the subsequent development of loose connective tissue on one hand, and those of dentine and bone on the other hand, I could ascertain that in the process of formation of their ground substance there is considerable analogy also in other respects.

The facts concerning the formation of ground substance of dentine and bone that I came to establish may be summed up as follows:

A compact dentino- and osteoblastic plasmodium arising from spongy mesenchyme consists of nucleo-endoplasmic areas and of ectoplasm common to the entire plasmodium (the synectoplasm of Studnička), in which argyrophil fibres are contained. These latter originate from the ectoplasm and are evidently a product of its condensation. With the beginning of the differentiation of the plasmodium, whose nucleo-endoplasmic complexes change into elements designated by me as pre-odontoblasts and pre-osteoblasts, the argyrophil fibres in the

<sup>1</sup> As I have shown (1928), the holes in the ectoplasm of the fibroblasts may not always be observed, and in some cases the ectoplasm of these cells appears to be continuous.

synectoplasm become considerably more numerous. In connexion with a further differentiation of pre-odonto- and pre-osteoblasts into odonto- and osteoblasts the fibres are transformed into collagen fibres and these pass directly into the ground substance of the dentine (exterior layer) and bone (coarse fibrillar). This direct transition of fibres gives another illustration and confirmation of the fact that is to be observed on suitably treated preparations, of a direct transformation of ectoplasm into the ground substance. With the further differentiation of the pre-odonto- and pre-osteoblasts in one part of the ectoplasm argyrophil fibres continue to form at once; while the other part remains amorphous and in this amorphous state changes into the ground substance, and argyrophil fibres arise from it only in the place and at the moment of this transition. In these early stages of formation of the ground substance of dentine and bone, it may frequently be observed that the differentiation of some of the pre-odonto- and pre-osteoblasts is cut short, and that they, as a whole, are changed into ectoplasm (into argyrophil and next into collagen fibres). The other elements achieve the highest degree of their differentiation, they are transformed into odonto- and osteocytes. In proportion to the exhaustion of the supply of primary odonto- and osteoblastic elements, and the destruction of those among them that have achieved the highest grade of their differentiation (the odonto- and osteocytes), elements of the second generation arise to take their place. These develop from indifferent cells located in the adventia of the nearest-lying vessels.

This is particularly to be observed in the stage corresponding to the end of the first comparatively short period of formation of the dentine (exterior layer) and bone (coarse fibrillar), and the beginning of the next more prolonged period of development of the circumpulpar dentine (interior layer) and lamellar bone.

The ectoplasm of the odonto- and osteoblastic elements belonging to this second generation differs from that of the elements belonging to the first generation in that it is all or nearly all amorphous. While changing directly from an amorphous condition into the ground substance of dentine (circum-

pulpar) and bone (lamellar) it becomes structured; but in place of the said transition, the fibres arising from this ectoplasm pass over the argyrophil phase, and appear from the very beginning as collagen fibres. The argyrophil fibres, which are to be observed in the dentino- and osteoblastic tissue in these subsequent stages, are chiefly connected with the adventitia of the smaller vessels. They are arranged in the ectoplasm of the undifferentiated adventitial cells that play the part of cambial cells.

Returning to the development of the loose connective tissue, mention must be made of the fact that in early stages of the development of mesenchyme, as observed by me on chicken embryos of 48-60 hours of incubation, the argyrophil fibres appear in the form of short separate or somewhat longer intertwining fine fibres lying corresponding to cells and their processes. A large number of argyrophil fibres may be observed at the places of contact between mesenchyme and the ecto- and endoderm,<sup>1</sup> as well as about the vessels. On the periphery of the chord and particularly of the neural tube to which the mesenchymal elements are close and adhere with their processes, the said fibres come to form a dense fibrillar membrane. In haematoxylin preparations of mesenchymal elements, as described by Studnička and Laguesse, particularly at somewhat later developmental stages, the prenuclear intensely stained endoplasm, as well as the homogeneous transparent ectoplasm, may most easily be distinguished. By comparing these preparations with sections impregnated with silver after Belschowsky's method, it can be ascertained that both the thicker and finer argyrophil fibres lie entirely in the ectoplasm of the mesenchymal elements connected syncytially. But I never observed any fibres arising as small clumps, rods, or grains, as Snessarew describes. Neither could I observe the 'strepto-

<sup>1</sup> It seems that the elements of primary mesostrome (the plasmodesms) are the first to achieve ectoplasmic nature, and change into argyrophil fibres; or more exactly they consist from the very beginning solely of ectoplasm. Since the mesostrome represents the presumptive mesenchyme, it is very probable that the argyrophil fibres, as described by Snessarew, are formed in it before immigration of the corresponding nuclei has taken place; in other words, before the appearance of real (cellular) mesenchyme.

bacillar' state of fibres described by the same author.<sup>1</sup> However, on some preparations of an unfavourable silver impregnation, all argyrophil fibres appear as grained chains. But as in the next sections, that were successfully impregnated, all the fibres have their usual character, this 'streptobacillar' form of the fibres must be considered as an artefact, which, as I have had occasion to observe, may appear also with other silver impregnation methods (for instance, that of Golgi).

In the next following stage in the areas of mesenchyme that develop into subcutaneous loose connective tissue, the cellular elements are distributed in planes. These elements continuing to divide mitotically remain after each division connected by ectoplasmic bridges that get larger and larger and finally become continuous. In this way gradually arise the symplastic lamellae, which in places are interconnected by bridges.<sup>2</sup> These lamellae consist of nucleo-endoplasmic areas and of common synectoplasm by means of which these areas are interconnected, and in which as before lie argyrophil fibres. Only these have become more numerous. This entitles me, on the ground of the data I have obtained concerning the early developmental stages of mesenchyme and loose connective tissue, to confirm in full the analogous observations made by Studnička and Laguesse.

But as regards the further development of the subcutaneous

<sup>1</sup> The arising of fibres from granules has also been described by Maximov and Bloom and before them by Golovinski.

<sup>2</sup> Laguesse mentions the possibility of formation of such lamellae from spongy mesenchyme as well as by the expansion and growth of cellular ectoplasm, which results in the intercellular spaces becoming smaller and even obliterated. Plenck (p. 377), quoting Laguesse, speaks of just this way of formation of the lamellae, and considers them as 'ground substance' that has been 'segregated' by the cells. Firstly, Laguesse himself (1921, p. 192) considers the formation of lamellae, that is connected with the division of the cells, to be of much greater importance, and correctly points out that by reason of continuous mitoses all the elements in the developing mesenchyme are continuously formed anew; and if the tissue 'a changé d'aspect, cela est dû beaucoup moins au changement de forme des éléments pré-existants qu'aux formes acquises peu à peu par les éléments nouveaux'. Secondly, as will be seen, the lamellae in the above developmental stage, containing only argyrophil fibres, do not as yet represent the ground substance.

loose connective tissue after it has obtained lamellar structure, my observations (made on mammals) diverge essentially from the analogous observations made by Laguesse.

Though it has not been possible, because of the lamellar structure of the tissue, to trace with equal clearness the separate details of this development as with dentino- and osteoblastic tissue, yet I can, on the basis of my preparations, affirm that, generally speaking, it takes place in the following way (Pl. 10, fig. 3).

Together, as well as in co-ordination with the gradual differentiation of the nucleo-endoplasmic complexes, the argyrophil fibres that lie in the synectoplasm of the symplastic lamellae change gradually into collagen fibres. Together with the amorphous remnant of the ectoplasm lying between them, and gradually acquiring structure, these collagen fibres form the ground substance which occupies the central part of every lamella. On their surfaces, as I could ascertain in tangential sections and especially in membrane preparations, the lamellae keep the structure of the symplast. This symplast, lying on the surface of the lamella, differs from the initial symplast, of which in the foregoing stage the whole lamella consisted, in that the synectoplasm which composes it contains a smaller number of argyrophil fibres; these latter are more slender, and in its major part the synectoplasm proves to be amorphous. Later on, this change of character of synectoplasm becomes more marked, and the synectoplasm appears as an extremely fine, almost entirely amorphous membrane, while the holes which were observed before become larger. In this stage argyrophil fibres appear in the lamellae along with collagen fibres. It is impossible to discern in this stage the exact localization of the argyrophil fibres. However, by analogy with corresponding stages of development of dentino- and osteoblastic tissue, one should consider that the synectoplasm acquires structure (in the form of argyrophil fibres) only in a small part, while most of it becomes amorphous. The argyrophil fibres arise from the part of the ectoplasm that changes into ground substance while being in an amorphous state, and this occurs only in the region and at the moment where the said transformation takes place. The direct transition of the ectoplasm into the ground substance

is expressed by the ectoplasm becoming thinner and by the formation of holes in it. As to the nucleo-endoplasmic areas of the symplast, some of them appear in this stage as elements of comparatively small size. They stain intensely, and show lively mitotic division. Other larger areas, possessed of a larger nucleus and probably more highly differentiated, stain weaker. Here are also to be found elements whose nuclei and ectoplasm stain picnotically. By analogy with what is observed in dentino- and osteoblastic tissue, it seems that the nucleo-endoplasmic complexes may possibly change as a whole into ectoplasm. In the next stage of development of the subcutaneous loose connective tissue, that chronologically is near to the moment of the animal's birth,<sup>1</sup> the holes in the ectoplasm become still more numerous and larger. The result is that the symplast lying at the surface of the ground substance acquires the form of a syncytium. However, the cellular elements, being a composite part of the syncytium, are not endoplasmic but diplasmic, while their ectoplasm appears as a transparent, entirely amorphous sheet. It is only in the elements dividing mitotically that the ectoplasm is not observed, these elements representing consequently in this condition purely endoplasmic cells.<sup>2</sup> The increase of the number and size of the holes in the ectoplasm continuing, results in the presence on the surface of the lamellae of subcutaneous loose connective tissue in the newborn animal (dog, rabbit), not of cells bound to a syncytium, but of isolated diplasmic fibroblasts. Their ectoplasm, which has the appearance of a large amorphous sheet, may in contradistinction to the autectoplasm of the mesenchyme cells containing argyrophil fibres, be designated as secondary autectoplasm.

<sup>1</sup> The time at which one or other of the described stages makes its appearance may shift according to different conditions. It depends on the species experimented with. Thus the mouse possesses under equal conditions a loose connective tissue of a lesser degree of differentiation than the dog or the rabbit, consequently in the mouse the corresponding stage sets in at relatively later periods.

<sup>2</sup> That in this case no ectoplasm can be observed may possibly be explained by the circumstance that it continues to be used for the formation of ground substance, while the restoration has ceased temporarily for the period of the mitosis.

At this stage a small number of argyrophil fibres may still be observed in the direct vicinity of the fibroblasts. Their localization and direction shows, however, that they lie not in the autectoplasm proper, but evidently at the place of its transition into ground substance. Thus, at this stage, the amorphous ectoplasm of the fibroblasts while becoming structured yet goes through the argyrophil phase. In all the later stages of post-embryonic life, in which the fibroblasts always (the condition of mitotic division excepted) possess autectoplasm,<sup>1</sup> the argyrophil fibres may be observed in the subcutaneous loose connective tissue principally around the small vessels, where they lie in the ectoplasm of the adventitial mesenchymal cells. These latter cells, as many authors affirm (Maximov, Zavarzin, and others), and as I myself (1929) had occasion to observe in an experimentally caused inflammation, play the part of a cambium,<sup>2</sup> at the expense of which in subsequent stages a new formation of the fibroblasts<sup>3</sup> takes place, these latter occasioning the further growth of the ground substance.

In the areas of the normal subcutaneous loose connective tissue that do not adhere directly to the blood-vessels, argyro-

<sup>1</sup> The hystiocytes as a rule are purely endoplasmic cells bereft of ectoplasm. But in new-born and very young rabbits and mice—in the latter even in adults—elements are to be found whose nucleus and endoplasm by all their properties testify that they belong to the hystiocytes, though at the same time they possess on their periphery a narrow, broken, but quite clearly visible ectoplasmic rim. Judging by certain signs these cells cannot be considered as fibroblasts that have recently undergone division and have acquired a temporary likeness to hystiocytes, and I consider them as feebly differentiated mesenchymal elements that change into hystiocytes and have not yet quite lost the ectoplasm.

<sup>2</sup> Chlopín (1925) states that in tissue cultures elements occur of various degrees of differentiation among fibroblastic cells.

<sup>3</sup> At a certain stage of development a replacement of the elements producing the ground substance, analogous to that just described for the dentino- and osteoblastic tissues, no doubt, takes place in the subcutaneous loose connective tissue as well. In the latter the said stage is more protracted, and consequently it is not possible to speak here with as much assurance of a replacement of elements. Testimony of such a gradually occurring change is however presented by the large number of amitotic divisions and degenerating fibroblasts that may be observed in the late embryonic and early post-embryonic stages.



phil fibres in post-embryonic stages as a rule are not observed. At these stages the fibres formed anew from the amorphous ectoplasm of fibroblasts changing into ground substance, in their development pass over the argyrophil phase; they make their appearance directly as collagen fibres. It is exactly these fibres that represent the mass of finest fibres ('Tramule', Renault) appearing in the preparations (when suitably treated) of the adult connective tissue; they arise in its 'amorphous' substance lying between the bundles of collagen fibres.

It follows from the above that the endoplasm of the fibroblasts (elements of the mesenchymal syncytium and those of the symplast), their ectoplasm and ground substance, must be considered as a series of formations changing immediately into one another.

I could follow sufficiently clearly the transformation of endoplasm into ectoplasm in dentino- and osteoblastic tissues. In these, certain nucleo-endoplasmic complexes, falling into a state of plasm- and caryo-pycnosis, change wholly into ectoplasmic substance, which in its turn is transformed into argyrophil fibres, while these later on metamorphose into collagen fibres. Although the living protoplasm that undergoes transformation may always be renewed, and though it is not at all necessary for the recognition of the fact of the transformation of protoplasm that "vom Protoplasten nichts, d.h. kein regenerationsfähiges Gebilde übrigbleibt", nevertheless we have here 'a transformation' of protoplasm even from the point of view of Petersen, whose opinion on this question has been cited above. Still clearer may be seen the direct change of endoplasm into ectoplasm in preparations of loose connective tissue, namely in the fibroblasts. For in these separate islets of endoplasm, not connected with its principal perinuclear mass, lie dispersed in the ectoplasm that otherwise has the appearance of an utterly structureless membrane.

The ectoplasm being most narrowly connected with the endoplasm imposes to such an extent as an integral part of the cell (i.e. of syncytium, of plasmodium), that to designate it as a product of 'secretion' would be entirely inadequate. As little suitable and as little conformable with the real relations as with the usual terminology would be to use such a designation for

the ground substance, considering that the latter arises immediately at the expense not of the endoplasm, but of the ectoplasm, which withal, for instance in the initial stages, is structured (it contains argyrophil fibres, which continue directly and change into collagen fibres of the ground substance). Thus, in accordance with other authors (for instance, Wassermann) I consider as before that the ground substance must be looked upon as a product exactly of the transformation of protoplasm and not as a product of secretion or 'segregation'.

Notwithstanding the existence of a direct transition between endo- and ectoplasm and between the ectoplasm and the ground substance, they may however be quite markedly defined. The distinguishing between endo- and ectoplasm, that morphologically are so clearly delimited, presents no difficulty at all. As to ectoplasm and ground substance, their distinctive features are as follows. The ectoplasm standing in immediate contact with the endoplasm contains in the initial stages of its development argyrophil fibres, which represent a condensed ectoplasmic substance as yet evidently not chemically, but only physically, transformed. Changing next into collagen fibres they come to form already an integral part of the ground substance. In this first relatively short period, in which the initial stages are included, the development of the supporting trophic tissue and of the ground substance occurs after the type that might be designated as *mesenchymal*. In a protracted second period, including all the subsequent stages (connected by gradual transitions with the earlier stages), the ectoplasm, which, as before, stands in direct contact with the endoplasm, appears to be amorphous; while the fibres which arise in it in the course of its transition into ground substance are formed directly as collagen fibres. In this second period the development of the supporting trophic tissue, as well as that of the ground substance, occurs according to another type, which may be designated as *desmal*. The principal feature characterizing the ground substance is the presence of collagen fibres. The substance lying between these fibres, which under the usual methods of treatment of the material appears amorphous,<sup>1</sup> represents the

<sup>1</sup> As pointed out by Renaut (1903) and Laguesse (1921), and as I had

rest of the ectoplasm that gradually, as fibres are formed from it, probably is transformed into a 'cementing substance'.

I cannot discover any deep fundamental difference between the formation of collagen fibres after the mesenchymal and after the desmal type, as in both cases the fibres arise from ectoplasm, which in both cases doubtless undergoes identical chemical changes. I have ascertained (particularly in my investigation of the dentinoblastic tissue) that even amorphous ectoplasm lying between argyrophil fibres is capable in certain stages, in which it is possessed of a certain density, the preparations being treated after Bielschowsky's method, of undergoing impregnation with silver and obtaining a greyish-black tint. In preparations impregnated with silver the tint of the fibres originating from ectoplasm varies from grey to intense black according to the degree of condensation of the ectoplasmic matter constituting these fibres. In the ectoplasmic substance during the initial phases of its metamorphosis into collagen the fibres that make their appearance are of sufficient condensation to permit the producing of black colour by silver impregnation (argyrophil fibres). On the other hand, in the same ectoplasmic, but evidently less dense, substance of the subsequent stages fibres are formed of so small a density that being impregnated with silver they become at most light grey in colour. When the Bielschowsky method is used they become visible only after

occasion to observe personally (1928), in preparations treated suitably the ground substance lying between the bundles of fibres in the loose connective tissue of the adult contains a great mass of the finest fibres. Wassermann, while speaking of this fact, considers it to be of particular importance as testifying to the formation of fibres taking place also outside the cells 'in der von den Zellen gelösten Grundsubstanz' (p. 635). The main point lies not in the question, where do fibres appear, but at the expense of what material does the formation in the later stages of this ground substance take place, which as it were 'has come out of contact with the cells' and in which the fibres continue to appear. Though Wassermann does not speak of it directly, yet it follows from the context that he tacitly recognizes that not alone the formation of fibres but also the increase of the substance from which these fibres originate may take place independently of the cells. As follows from the above, the finest fibres contained in the 'amorphous' ground substance are formed at the expense of the ectoplasm, which also in the adult connective tissue the fibroblasts continue to produce.

having assumed the properties and the staining reaction of collagen fibres. The absence of the argyrophil phase is due to this. In connexion with this I must state that I, like Heringa and Hooft, cannot share the opinion of those authors (Renaut, Mallory, Nageotte, Doljansky and Roulet) who consider the difference between the argyrophil and collagen fibres to depend only on their calibre. According to these authors the separate fibres in which the bundles of collagen fibres divide or may be divided become again argyrophil, while the argyrophil fibres, when uniting into bundles, lose their argyrophily and become collagenous (Doljansky and Roulet). As a matter of fact the argyrophil fibres when changing into collagen do not fuse together; on the contrary they split and the collagen fibres always prove to be finer than the argyrophil ones. I believe that collagen fibres cannot be transformed into argyrophil fibres under any conditions.<sup>1</sup>

Endoplasm, ectoplasm, and ground substance may be considered as a series of formations possessed of decreasing vital properties. Such a point of view would coincide with Weigert's opinion, who interpreted the formation of the ground substance as a katabiotic process. It may be that in the series mentioned the place last in order must in a certain sense be allotted to the interstitial liquid, into which possibly the ground substance changes without there being any distinct limit between them. Such a point of view might to a certain degree reconcile the contradiction existing between the opinion of Laguesse, which has found support in the present work, concerning the lamellar structure of the loose connective tissue, and the point of view of Petersen, who denies the existence of such a structure.

In conclusion I should like to mention certain data which are usually cited in numerous works of the authors who have studied the problem of the formation of ground substance on tissue cultures. These studies (for example the recently published work of Doljansky and Roulet) are mostly based upon the cultivation of feebly differentiated or undifferentiated cells (reticular or mesenchymal) with a subsequent treatment of the

<sup>1</sup> The interesting experimental results of Heringa and Hooft (1926 and 1933) will be dealt with elsewhere.

preparations after the method of Bielschowsky. It is the question of the intra- or extracellular formation of the argyrophil fibres that is discussed here in most detail. All these authors designate as cells the nucleo-endoplasmic complexes, and therefore, as is quite natural, they find in their preparations an extracellular formation of fibres. Stating the negative fact that the argyrophil fibres do not arise inside the endoplasm<sup>1</sup> certainly does not explain the origin of the fibres and the ground substance, and is no positive proof of either of the theories. However, on the strength of some indirect data and considerations, most authors incline towards accepting the theory which admits the possibility of the formation of argyrophil fibres (in so far as they do not mix them up with fibrin fibres) from nutritive plasm (interstitial liquid) under the influence of a hypothetical ferment secreted by the cells.

As an example of such indirect data, one may cite the circumstance, seemingly testifying against the ectoplasmic theory, that the direction and arrangement of the argyrophil fibres appearing in the tissue culture, particularly in its central zone, does not coincide with the direction and arrangement of the cells. The proofs positive of the ectoplasmic theory, presented by Studnička, Laguesse, Zavarzin, Wassermann, and others, are not taken into consideration as well as the fact, stated by myself already in 1924, that in the dentinoblastic cells together with the endoplasm the ectoplasm may clearly be seen, and that the endoplasm of these cells continues into the Tomes processes, while the ectoplasm immediately passes into the ground substance. The fact I established in 1928, concerning the presence of ectoplasm in the fibroblasts of loose connective

<sup>1</sup> A confirmation of this circumstance might be of some use, as up to now certain authors keep to other opinions. As for instance, Wassermann, referring to the investigations made by Studnička, Tello, Hartmann, and Orsos, admits (1929, p. 620) that the first fibres are formed in the endoplasm. Studnička has now, in his recently published work (1933), discarded his former point of view. If the fibre passes through the cellular body (Wassermann, p. 617), it does not necessarily mean that it is the endoplasm it passes through. On well-fixed and contrastingly stained preparations it is possible by focussing to ascertain that in these cases the fibres do not lie in the same plane as the endoplasm.

tissue in post-embryonic stages, which quite decisively speaks in favour of the ectoplasmic theory, is equally not taken into consideration.

A detailed study of the process of fibre formation in conditions of tissue culture will be the subject of my next investigation. However, on the basis of literary data as well as on the study of the, as yet but few, preparations at my disposal, I consider it most probable that the formation of the fibres *in vitro* takes place exactly in the same way as described in the present work for the formation of the fibres *in vivo*.

What the authors consider in tissue cultures as ground substance is actually the syncytoplasm<sup>1</sup> of the syncytium (symplast), growing along the surface of the nutritive plasm and including as well the nucleo-endoplasmic areas (cells). In this syncytoplasm the argyrophil fibres that are formed in it and from it, extend at the moment of their formation in this or that direction, presumably influenced by mechanical forces of strain and pressure ('Zug und Druck'). In some areas of the culture, for instance in the zone of intense growth, this direction may coincide with the direction and disposition of the cells, while in other areas, particularly in the more central ones of the culture, the fibres may take another direction. Similar relations may also be observed, as is described above, in the formation of argyrophil fibres in the dentino- and osteoblastic (radial and tangential fibres) as well as in the loose connective tissue. Tissue cultures maintain on their free surface the syncytial (symplastic) structure also in those cases when the authors managed to obtain a (be it only partial) transformation of the argyrophil fibres formed in the culture into collagen fibres—in other words to obtain the formation of a real ground substance.

If in such cultures fibroblasts, possessed of a secondary autectoplasm and analogous to those I have found in the loose connective tissue in post-embryonic stages, are not observed, this refutes nothing. One could expect their appearance if the tissue culture is lead up to the stage of development in which the fibroblastic syncytium (symplast) divides into separate more or less highly differentiated fibroblasts. Whether this can be

<sup>1</sup> A necessary feature of the ground substance are the collagen fibres.

attained in the conditions of the culture remains yet to be studied.

I find it incumbent to mention shortly such considerations pertaining to the formation of ground substance as Wassermann mentions in his compendium. On p. 651 he remarks: "von Bedeutung ist dagegen wieder Iasswoins Feststellung, dass das Ectoplasma der Fibrocyten im gewöhnlichen Zustand des Bindegewebes nicht nur durch keinen direkten Übergang, sonder nicht einmal durch einfache Berührung mit der Grundsubstanz in Beziehung steht." It is quite true that in the work quoted (1928, p. 148) I make such an affirmation, but there I speak of endoplasm, not of ectoplasm. After this seeming misprint, utterly changing the meaning of my words, Wassermann continues: "der Bildungsprozess ist beim erwachsenen Tier eben in der Hauptsache abgeschlossen und dazu gehört nach der von uns ganz in Übereinstimmung mit Iasswoin entwickelten Anschauung über die Entstehung der Grundsubstanz, dass diese, wenn der Vorgang die letzte Stufe wie beim Bindegewebe, dem Knorpel, Knochen und Dentin erreicht, gegenüber den Zellen selbständig wird." This is a point of view I cannot accept. It is indubitable that in the loose connective tissue of an adult animal a constant replacement of fibres occurs, and consequently the fibres are constantly formed anew. If in the loose connective tissue this process may not be proved, it has already for a long time been known to exist, for instance, in bone tissue and studied in detail. Now as before (1924, 1928, 1933) I am of the opinion that the formation of fibres may take place so long as the cells provide or have provided the necessary ectoplasmic material. So long as the material is being provided, the cells keep immediate contact by means of their ectoplasm with the ground substance. If the ground substance loses contact with the cells—and this takes place only in the case of the transformation of the entire cell into ectoplasm and further into ground substance, or in case of their physiological or pathological degeneration—the formation of fibres ceases after the ectoplasmic material (ectoplasm that changes into ground substance) contained in the amorphous ground substance has been used up.

The influence that the cells (the nucleo-endoplasmic complexes) have on the formation of the ground substance and of the fibres, which may be recognized by microscopical investigation, is expressed in that these cells deliver the ectoplasmic material, whose properties change parallel to the change of the properties (the grade of differentiation) of the nucleo-endoplasmic complexes. A differentiation of the ectoplasm independent of the nucleo-endoplasmic complexes should not be admitted, nor an independent quantitative increase. In those cases in which the differentiation of the nucleo-endoplasmic complexes stops at a certain stage, the differentiation of their ectoplasm stops at the same stage.

The observations described above may be summed up as follows:

In the course of the development of the mesenchyme into subcutaneous loose connective tissue lamellae appear that primarily represent symplasts, and consequently consist of nucleo-endoplasmic areas and common ectoplasm (synectoplasm), which contain argyrophil fibres.

The course of the further development of these symplastic lamellae, during which a ground substance containing collagen fibres is formed in them, may, according to the mode of formation of the ground substance, be divided into two unequal periods: the first, short period, including the initial stages of development, and connected to it by gradual transitory stages, the second period, more durable, including all the subsequent stages.

In both periods the ground substance consisting of collagen fibres and the 'cementing' substance is formed out of ectoplasm by the direct transformation of the latter.

In the first period the collagen fibres are preformed as argyrophil fibres arising in the ectoplasm itself.

In the second period collagen fibres are formed from ectoplasm that undergoes transformation into the ground substance while being in an amorphous state; these fibres originate directly as collagen fibres.

The 'cementing' substance is an amorphous and evidently a transformed remnant of the ectoplasm after the formation of collagen fibres from it has taken place.



In the first period the ground substance is formed after the type that might be designated as 'mesenchymal', in the second period it takes place after the type that, in contradistinction to the before-mentioned, may be designated as 'desmal'.

The connective tissue lamellae that are formed in the course of development from the primary mesenchymal symplasts (the latter containing solely argyrophil fibres) and in whose central area a ground substance containing collagen fibres is already present, are at the beginning still covered on their surface by the symplast. Later on this symplast changes into an endo-ectoplasmic syncytium, which then, in its turn, divides into separate diplasmic fibroblasts. The modifications of the properties of the ectoplasmic substance that take place here are undoubtedly connected with the corresponding modifications of the degree of differentiation<sup>1</sup> of the nucleo-endoplasmic complexes, forming an integral part of the above-mentioned formations beginning with the mesenchymal symplast and ending with the separate fibroblasts.

In conclusion, I wish to express my thanks to Prof. Goodrich for revising the English text of this article.

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<sup>1</sup> The differentiation of cellular elements does not occur simultaneously; in every developmental stage the tissues contain cells in different stages of differentiation. In the adult organism, too, there remains in the loose connective tissue a reserve of non-differentiated ('mesenchymal') elements. In animals whose connective tissue remains on a lower level of development (for instance, in white mice), these cells are diffusely dispersed everywhere in the connective tissue. On the other hand, in animals whose connective tissue is more highly developed (for instance, in dogs) the undifferentiated cells prove to be concentrated principally about the smaller blood-vessels (adventitial cells, pericytes).

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## DESCRIPTION OF PLATE 10

FIG. 1.—Loose connective tissue of an adult mouse. Tangential section. Fixation after Laguesse. Paraffin. Staining with iron haematoxylin after M. Heidenhain. Diplasmic structure of fibroblast is to be seen. Imm., oc. 2.

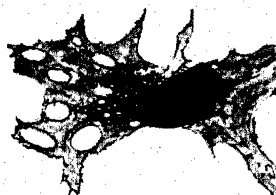
FIG. 2.—Fibroblast from loose connective tissue of an adult rabbit. Film preparation. Imm., oc. 3.

FIG. 3.—Diagrams: (a) spongy mesenchyme. Nucleo-endoplasmic complexes are bound by a common synectoplasm, in which argyrophil fibres are lying; (b) loose connective tissue in development: the central part of the lamellae consists of ground substance, containing collagen fibres. The surface of the lamella is covered by a symplast, composed of nucleo-endoplasmic complexes and synectoplasm, which contains argyrophil fibres; (c) loose connective tissue in development. The next stage. The ground substance containing collagen fibres in the central part of the lamella. On the surface—diplasmic syncytium. At the place of transition of ectoplasm into the ground substance argyrophil fibres are still to be perceived; (d) formed loose connective tissue: on the surfaces of the lamellae of the ground substance are situated in the form of bas-reliefs separate diplasmic fibroblasts. Their ectoplasm remains immediately bound to the underlying ground substance, on which they lie, while continuing to be transformed into the latter. The fibres formed here arise, as a rule, directly as true collagen fibres.





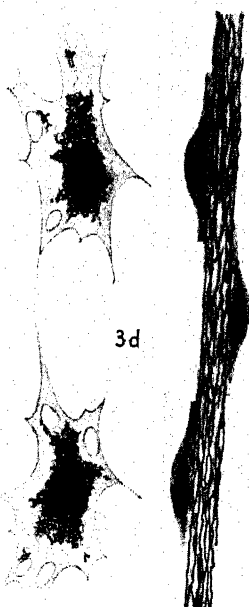
3a



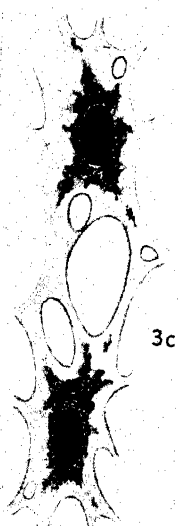
2



3b



3d



3c



# Note on the Behaviour of certain Cell Inclusions during Mitosis in Tenthredinidae.

By

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With Plate 11.

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### I. INTRODUCTION, TECHNIQUE, ACKNOWLEDGEMENTS.

THE occurrence of extranuclear bodies in cells has been recorded for many species of animals. Some have been identified as mitochondria and others as Golgi bodies, but in a large number of cases it has not been possible to identify them or to assign to them any well-defined behaviour or function. Such bodies are usually referred to as 'inclusions', 'chondriosomes', 'chromatoid bodies', or by a variety of indefinite names.

Various cytological workers of this department have noticed such bodies in the Tenthredinidae (e.g. Sanderson, 1932), but did not enter into details of individuality and behaviour. In recent work on the parthenogenetically arrhenotokous Tenthredinid, *Thrinax mixta*,<sup>1</sup> the repeated occurrence, in a well-defined space and time relationship to mitosis, of chromatoid bodies in the blastoderm of a partheno-produced individual, induced a close study of these inclusions.

<sup>1</sup> The nomenclature employed for Tenthredinidae mentioned in this paper is that of Enslin, 'Die Tenthredinoidea Mitteleuropas', 1912.



The cytological material was obtained from virgin females of *Thrinax mixta*. The eggs, laid in the still uncurled fronds of *Aspidium filix-mas*, were allowed to develop from periods ranging from 1 to 3 days and were afterwards fixed in Zenker's fluid, cleared in cedar-wood oil, and embedded in paraffin wax (M.P. 52° C.). Sections were cut at 5  $\mu$  and stained according to Heidenhain's iron haematoxylin technique. A modification of the usual practice was introduced, namely, only 4 hours treatment in 5 per cent. iron alum solution followed by treatment in iron haematoxylin for the same period. This modification was introduced because the heavy black deposit of stain obtained by the long method obscured the individuality of the chromosomes in metaphase plates and also prevented the ready distinction between the chromosomes and the inclusions, which also stain black. By the short method the chromosomes stain blue and the inclusions black.

Our findings have been made incidentally during researches on parthenogenesis of Tenthredinidae, which researches have been assisted by grants from the Department of Scientific and Industrial Research.

## II. RESULTS.

The bodies were first detected at full metaphase during mitosis (fig. 1, Pl. 11). They are four in number, and are seen to lie more or less symmetrically, two on each side of the plate, and situated about midway between the plate and the poles. They have a spherical form and a diameter of about 1.25  $\mu$ . Like the chromatin they stain darkly, but it is easy to distinguish between them and chromatin, for the latter is of a bluish, rough granular texture quite distinct from the black, homogeneous, smooth 'oily' appearance shown by the bodies. Individual chromosomes measure about 0.2  $\mu$  long. With the incidence of anaphase the bodies, facing one another on opposite sides of the plate, approach and coalesce more or less completely into two masses which lie in the plane formerly occupied by the plate (figs. 2 and 3, Pl. 11). With further continuance of the anaphase these two masses approach one another in the plane of the plate and at right angles to their former direction of

motion (fig. 4, Pl. 11), the culmination of the fusion process being the production of a single body occupying a central position in the spindle figure (fig. 5, Pl. 11). During the ensuing telophase the single mass streams out after the chromatin in the direction of the poles of the spindle (figs. 6 and 7, Pl. 11). The onset of the cytoplasmic split into daughter cells finds the stream still flowing, but giving the appearance of being diffused into the cytoplasm without any association with the chromatin. The further history of the inclusions is unknown, but it may be mentioned, however, that in resting cells much smaller bodies than those under discussion are found in the cytoplasm, usually from two to four in number, but the relationships of the two kinds cannot be given with certainty at present.

### III. DISCUSSION.

The account of kinesis given above is based on sections of blastoderm produced parthenogenetically and therefore male and 'haploid' in constitution. Bodies similar in number, appearance, and relationship at metaphase have been observed in other parthenogenetic saw-flies, viz. spermatogonia of *Trichiocampus viminalis*, another arrhenotokous species, and in the oogonia of *Hemichroa rufa*, a thelytokous species, so that it cannot be said that they have a specific or sexual significance.

In the spermatogenesis of certain scorpions E. B. Wilson (1931) recorded inclusions to which those described here bear a certain amount of resemblance, and Wilson proved that they produced the axial filament of the sperm.

Bělař, in his researches on mitosis in *Chorthippus* (*Stenobothrus*) *lineatus* (1929), investigated the kinesis of mitochondria in its association with mitosis, and our figures almost parallel his in point of space relationship and time sequence. This is illustrated in the present paper by figs. A to D, Pl. 11 (drawn from Bělař's paper), which refer to interkinesis and the second maturation division in the male of *Stenobothrus*.

The resemblance being strong, one is inclined to make comparisons and suggest three obvious questions: (1) Are the bodies

mitochondria, as in Bělař's material? (2) Are they external to the spindle? (3) Is the fate the same in both cases?

With regard to the first it should be observed that the bodies are not thread-like, while in Bělař's material the mitochondria are definitely so. The true comparison is between a single body in our material and an aggregation of thread-like bodies in *Stenobothrus*. The difference does not appear to be due to the techniques employed. In the resting stage in *Stenobothrus* (fig. D, Pl. 11), however, Bělař figures the mitochondria as a large spherical mass with a remarkable resemblance to the inclusions in our material.

These inclusions are unlike mitochondria in that they are not thread-like or in dots, but it is not thereby concluded that they are not mitochondria. More convincing evidence of the 'mitochondrial' nature of the inclusions is obtained from the facts of technical treatment. The investigations for which the sections were originally prepared did not demand the use of such methods as those of Benda and of Bensley, and hence conclusive evidence has not yet been established—at least as far as such methods would clinch the matter. As is well known, bichromate fixatives in conjunction with Heidenhain's iron haematoxylin are ideal for mitochondria (cf. Regaud's method), and while Zenker's fluid, which we employed, is not quite the best bichromate fixative, results can be obtained with it. Material fixed in Bouin's picro-formal, which is not a mitochondrial fixative, shows the inclusions, but as minute bodies.

Fixation technique strongly suggests therefore that we are dealing here with mitochondria and chondriokinesis.

With regard to the appearance at telophase, it should be observed that the spindle fibres themselves give the semblance of a streaming effect; but in the present case this is due to something else, for material fixed in Carnoy's fluid, which does not reveal the bodies, shows the fibres alone, and the density is not at all so great as it is in the case of Zenker-fixed material.

The differences between the bodies and chromatin have already been dealt with, but we should like to lay stress on these differences because serious confusion might result from the spatial position of the bodies at metaphase giving the mistaken

impression of sex chromosomes moving to the spindle poles precociously. The danger of confusion is particularly great in the case of material fixed with Bouin or other fluids, which, while not able to reveal the 'mitochondria' so well as bichromate fixatives, do not sufficiently conceal their presence, but show them much reduced, often to a size comparable with that of a chromosome. We suggest it as a possibility that observations of 'precession' of chromosomes in maturation divisions, supposedly indicative of sex chromosomes, could in reality relate to bodies of the nature we describe. Moreover, owing to the position of the inclusions at early anaphase, plates in polar view often contain one or two of the inclusions at a very slightly different focus, and this is a possible source of confusion because the bodies might be interpreted as belonging to the chromosome complex, while their striking morphological characteristics might cause them to be regarded as of sex significance.

Regarding the question of the spatial position of the bodies, it is clear from profile and polar views that they lie on the spindle surface but are not part of it, and that the chromatin masses pass completely between them. We have in this an explanation, on mechanical grounds, of their kinetic behaviour; it is due to constriction changes in the form of the spindle during mitosis, during which the bodies play a passive part. This agrees with the interpretation put by Bělař on the appearances shown by the mitochondrial aggregations at mitosis in *Stenobothrus*.

As has been mentioned earlier, we have not been able to assign any definite function to the inclusions nor to associate them with the production of any particular structure, and it would seem, from his figures, that Bělař also did not trace their fate, as Fig. D, Pl. 11, given in our plate, is his last figure of them. It should, however, be mentioned that Bělař was not principally concerned with elucidating the whole history of the mitochondria, but was utilizing the appearances shown by their aggregations as a key to the causal mechanics of mitosis.

#### IV. SUMMARY.

1. Four conspicuous rounded bodies, lying symmetrically disposed, one on the surface of each quadrant of the spindle,

have been found during the metaphase of blastoderm cells developed from the partheno-produced (male) eggs of *Thrinax mixta*, a parthenogenetically arrhenotokous species of Tenthredinidae.

2. During the process of cell division they ultimately form a single mass in a position formerly occupied by the centre of the spindle, and the material composing this mass streams out towards either pole and appears to be more or less equally shared between the two daughter cells. The movements of the inclusions appear to be due to changes in the form of the spindle.

3. Their fate is unknown, as is their function.

4. Such bodies and their behaviour do not appear to have been recorded before, but in certain respects they behave like aggregations of thread-like cell inclusions in *Stenobothrus lineatus* which Bělař calls mitochondria; that they are mitochondrial is suggested from the technique which revealed them.

5. It is suggested that such bodies might easily be mistaken for sex chromosomes.

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#### EXPLANATION OF PLATE 11.

Figs. 1 to 8 are free-hand made directly from object, and checked for magnification and proportions by means of a camera lucida (Spencer No. 5 Microscope,  $\frac{1}{18}$  inch oil immersion parachromatic Watson objective, 10× Spencer planoscopic ocular). Figs. A to D are drawn for comparison from Bělař’s Monograph (1929).

Fig. 1.—Full metaphase; blastoderm mitosis in *Thrinax mixta*. *b.*, bodies under discussion; *ch.*, chromatin.

Fig. 2.—Early anaphase.

Fig. 3.—Late anaphase.

Fig. 4.—Onset of telophase.

Figs. 5-7.—Telophase.

Fig. 8.—Completion of mitosis.

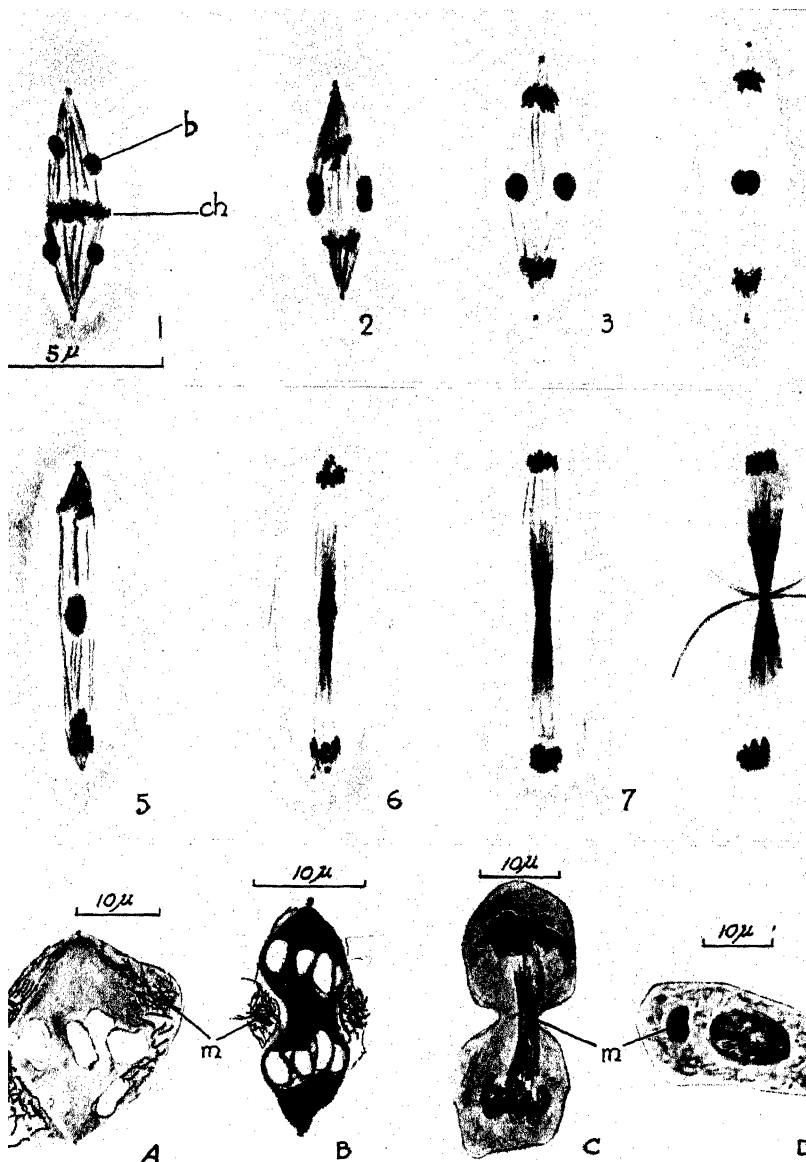
Fig. A.—Metaphase; mitosis of second spermatocyte in *Stenobothrus lineatus* m., mitochondria.

Fig. B.—Anaphase.

Fig. C.—Onset of telophase.

Fig. D.—Completed division; resting stage with mitochondria.









# The Spermatogenesis of *Stenophylax stellatus*, Curt. (Trichoptera).

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With Plates 12 and 13.

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## I. INTRODUCTION.

THE oogenesis of *Stenophylax stellatus* has been described in a previous contribution (5). The present study was undertaken, in the first place, to follow the prophase changes of the meiotic division, and to obtain chromosome counts which could be compared with counts in the female tissue. The results of these counts were mentioned in the paper on oogenesis, but spermatogenesis was not described. During these preliminary observations it was noted that the cytoplasmic inclusions of the spermatocytes were preserved in Flemming (without acetic) preparations. As the form of the Golgi material seemed to differ widely from that of other insects, and as the cytoplasmic inclusions of the male germ-cells of the Trichoptera do not appear to have been studied previously, it was decided to study the cytoplasmic inclusions and the stages of spermateleosis in *Stenophylax*.

Owing to the small size of the cells and chromosomes a

detailed study of chromosome structure and behaviour was not attempted, but an account of the main features of the nuclear changes during spermatogenesis is given.

Lutman (8) has described the spermatogenesis of *Platyphylax designatus*; and more recently Pchakadge (9) and Kleingstedt (7) have dealt with the gametogenesis of the Trichoptera. Lutman's observations were dealt with in the present writer's paper on the oogenesis of *Stenophylax*, but the papers by Pchakadge and by Kleingstedt were not mentioned; these will be referred to, where necessary, in the present contribution.

## II. MATERIAL AND METHODS.

Material was obtained from larvae and from a small number of pupae of *Stenophylax stellatus* collected from the Braid Burn, near Edinburgh. The structure of the testes, the nucleus and the nuclear changes of the spermatogonia, spermatocytes, and early spermatids were studied in the spring and summer of 1933; the work on the cytoplasmic inclusions and on spermateleosis was carried out in the spring and summer of 1934.

Material was fixed in the fluids of Flemming, Tellesynczky, and of Bouin. For the study of the Golgi bodies the method of Mann-Kopsch was found to be satisfactory. Both the Golgi bodies and the mitochondria were usually shown in material fixed in strong Flemming (without acetic) and subsequently stained in iron haematoxylin. Good results were also obtained with material fixed in Flemming (without acetic), and then placed in potassium bichromate for a few days; the sections were stained in acid fuchsin and methyl green. The testes of certain larvae were treated according to Feulgen's technique for the identification of chromatin.

I was aided in the work carried out in 1933 by Mr. A. R. Melville, B.Sc., whom I thank for collecting material, for preparing some of the slides, and for help on observations on the nucleus and on the general structure of the testes. I wish to thank Professor J. H. Ashworth, F.R.S., for reading the manuscript. The work was aided by a grant from the Earl of Moray Endowment of the University of Edinburgh.

## III. OBSERVATIONS.

## 1. Spermatogonia and Spermatocytes.

(a) The Nucleus.—The testes of the youngest larvae dissected contained both primary and secondary spermatogonia. The primary spermatogonia and their nuclei are large, and the latter contain a prominent, deeply stained nucleolus, and granules of chromatin arranged on a network; each cell is accompanied by a large epithelial nucleus containing darkly stained chromatin granules (fig. 1, Pl. 12). The primary spermatogonia divide to give rise to the secondary spermatogonia which occur in cysts surrounded by a cyst-wall; the latter appears to have but one epithelial nucleus, and the mature cysts to contain thirty-two spermatogonia. The epithelial nucleus is, at first, situated at one side of the cyst (fig. 44, Pl. 13). Later, when the first spermatocytes are formed the nucleus appears to spread out so as to surround the cyst almost completely (fig. 45, Pl. 13). Many of the secondary spermatogonia are in division; the nuclei of those in the resting stage contain an irregularly shaped nucleolus which is deeply stained, the chromatin granules, however, are small and are more faintly stained than in the earlier cells (fig. 2, Pl. 12).

In the part of the testes next to the sperm duct, and in testes from older larvae, many of the cells are in the growth stage of the first spermatocyte. The nucleus is large and contains a prominent nucleolus and small irregularly shaped masses of chromatin arranged on a network; both network and chromatin are more faintly stained than is the nucleolus (fig. 45, Pl. 13). In the older larvae this region is followed by spermatocytes in the prophase of the meiotic division.

The chromatin granules spread out on the network to form thin leptotene threads, which at first have a beaded appearance, but later become thicker and more deeply stained. A nucleolus is present and lies towards one side of the nucleus; it tends to become elongate when the leptotene threads are formed (fig. 3, Pl. 12). The threads are now gathered into a loose knot towards one pole of the nucleus (fig. 4, Pl. 12); later a dense synizetic

knot is formed from which a few threads project (fig. 5, Pl. 12). The nucleolus can usually be identified, as it is often situated at some distance from the chromatin knot. In the next stage the threads of the synizetic knot loosen to form thick pachytene threads; the nucleolus is still present but is smaller and, sometimes, more difficult to identify than in the earlier phases (fig. 6, Pl. 12). The pachytene stage is followed by the diplotene; after the latter stage the chromatin threads are stained faintly. Later the threads are more deeply stained and give rise to chromosomes, which become arranged on the metaphase plate of the meiotic division. During the diffuse stage a deeply stained nucleolus is present (fig. 7, Pl. 12). Numerous counts of polar caps gave the haploid number as thirty chromosomes (fig. 8, Pl. 12). Centrioles were not observed during the growth stage and prophase; this is probably due, in part, to the granular mitochondria present in most of the Flemming preparations, thus making identification of the centrioles extremely difficult. During both spermatocyte divisions the centrioles were identified in many cells (fig. 9, Pl. 12).

After the first division of the meiotic phase (figs. 9 and 10, Pl. 12) the chromosomes of the recently formed second spermatocytes become clumped so that it is difficult to make out any detail (fig. 11, Pl. 12); later the chromosomes reappear, the second division takes place, and the spermatids are formed.

Material containing primary spermatocytes in the growth phase and in stages of the meiotic prophase was treated according to Feulgen's technique. The chromatin granules and threads gave the correct reaction, but the nucleolus remained unstained in these preparations.

(b) The Cytoplasmic Inclusions.—An examination of Mann-Kopsch preparations showed that the Golgi material of the majority of the primary spermatogonia consists of a deeply impregnated mass situated at one side of the nucleus; in some cases this mass appears as a ring-shaped structure (fig. 12, Pl. 12), while in others it has the form of an irregularly shaped homogeneous body. In certain cells appearances indicate that the mass of Golgi material breaks up into granules and thick

rod-shaped bodies (fig. 13, Pl. 12), and that later these elements become distributed through the cytoplasm (figs. 14 and 15, Pl. 12). During the metaphase of the primary spermatogonia the Golgi bodies were identified as granules and thick rods situated in the vicinity of the spindle (fig. 16, Pl. 12). From these observations it is concluded that, prior to cell division, the mass of Golgi material breaks up into elements, that these become distributed through the cytoplasm, and that during the early stages of cell division they are scattered through the cell in the neighbourhood of the spindle; later they tend to pass towards the spindle poles, and are thus distributed with approximate equality between the two resulting cells.

The mitochondria of the primary spermatogonia were well shown in sections stained with acid fuchsin and methyl green. At the stage when the Golgi material is clumped at the side of the nucleus granular mitochondria occur scattered through the cytoplasm, but chiefly in the region surrounding the nucleus (fig. 17, Pl. 12). During the metaphase the mitochondria consist of fine granules distributed through the cell; they tend to form a few small clumps. The Golgi elements are shown close to the spindle (fig. 18, Pl. 12).

The Golgi material of the secondary spermatogonia is greatly reduced in amount; during the resting stage it occurs as a deeply impregnated body situated close to the nucleus (fig. 19, Pl. 12). The behaviour of the mitochondria and Golgi elements during the division of the secondary spermatogonia appears to be closely similar to the behaviour of these bodies during the division of the primary spermatogonia. The presence of groups of Golgi elements at the spindle poles during the anaphase and telophase suggests that the Golgi bodies precede the chromosomes in their passage to the poles (figs. 20 and 21, Pl. 12).

During the growth stage the Golgi material of the primary spermatocytes increases in amount, and in the younger cells the elements are clumped at one pole of the nucleus (fig. 22, Pl. 12). Later the rods and granules forming this mass move slightly apart (fig. 23, Pl. 12); the elements continue to separate (fig. 24, Pl. 12), and two clumps of Golgi bodies are formed which move

apart but do not pass to opposite poles of the nucleus (figs. 25 and 26, Pl. 12). The mitochondria are in the form of a loose clump of fine rods and granules situated close to the Golgi material; in some cases, however, they appear to have spread out slightly, but still remain in the neighbourhood of the Golgi elements. During the growth stage the mitochondria were often observed to surround an area which stained more deeply than the surrounding cytoplasm.

In the majority of cells undergoing the prophase changes for the meiotic division the Golgi material is in two masses (fig. 27, Pl. 12), but in some cases the bodies forming the original mass have not yet separated, while in others the two groups are still in contact. During the leptotene stage granular and filamentous mitochondria occur at the same side of the nucleus as the Golgi material. In the later phases the mitochondria spread out through the cytoplasm, but remain most numerous in the vicinity of the Golgi material.

The two masses of Golgi material break up and the elements separate, so that at the metaphase the latter occur chiefly in the cytoplasm adjacent to the poles of the spindle. The mitochondria come to lie at the side of the spindle (fig. 28, Pl. 12); later they come together to form two fairly compact masses beside the spindle, and thus each mass is divided into two during the telophase (fig. 29, Pl. 12).

From the above account it will be seen that the division of the Golgi material into two groups is preparatory to its subsequent distribution to the second spermatocytes, and that by this means there will be a group of Golgi bodies in the vicinity of each pole of the spindle. The spreading out of the mitochondria through the cytoplasm results in their subsequent position beside the spindle during the metaphase. Thus the Golgi elements and mitochondria come to be distributed with approximate equality to the second spermatocytes.

Two masses of Golgi material are present in the secondary spermatocytes (fig. 30, Pl. 12). During the division of the second spermatocytes the mitochondria and Golgi bodies are distributed in the same manner as in the division of the primary spermatocytes (figs. 31 and 32, Pl. 12).

## 2. Spermateleosis.

The chromatin of the young spermatid stains but lightly, and the nucleolus is a darkly stained spherical body (fig. 33, Pl. 13). As the spermatids elongate the nucleolus disappears and the nucleus stains faintly and more or less homogeneously in iron haematoxylin (fig. 34, Pl. 13). The mitochondria, in the form of granules and filaments, are clumped towards one pole of the nucleus; the Golgi material is present as rounded bodies or granules (fig. 33, Pl. 13). At the next stage the filamentous mitochondria form a tangled mass which moves from the pole of the nucleus towards the tail region. The Golgi material has increased in amount, and is present as three or more comparatively large, irregularly shaped masses situated in the cytoplasm, chiefly posterior to the nucleus. Meanwhile, the axial filament has made its appearance, and a centriole, which is in all probability double, can now be identified close to the nuclear membrane. In many cases a second granule is in contact with the nucleus (figs. 34 and 35, Pl. 13). It is probable that the second granule is a post-nuclear body, this matter, however, is discussed on p. 323. The mitochondrial mass now moves towards the axial filament, and becoming more compact, finally completely surrounds the filament (figs. 35, 39, and 43, Pl. 13).

Many of the preparations showed that a mass of Golgi material was present in the vicinity of the anterior pole of the nucleus of the younger spermatids, and that at a slightly later stage Golgi bodies are present in the tail region only (figs. 37 and 38, Pl. 13). Further investigation showed that the sequence of events is as follows: One mass of Golgi material comes to lie near the nucleus, and at the time when the mitochondria are moving towards the axial filament, this mass begins to migrate towards the anterior pole of the nucleus (figs. 34 and 37, Pl. 13). It comes in contact with the nuclear membrane (figs. 38 and 42, Pl. 13), and later moves away from the latter through the cytoplasm. In favourable preparations a small granule can be identified in contact with the nuclear membrane in the position formerly occupied by the mass of Golgi material; this granule is the developing acrosome (figs. 40 and 41, Pl. 13). As the mass



of residual Golgi material passes towards the tail region the acrosome increases in size. In all cases the young acrosome was deeply stained, so that it did not show differentiation into a vesicle and a granule; furthermore, its presence was not detected until the Golgi remnant had moved away from the nuclear membrane. It is concluded that the acrosome is formed under the influence of the Golgi material, but owing to its small size and to the manner in which the Golgi material surrounds it, it is impossible to observe the early stages in detail. Appearances indicate that the acroblast does not come in contact with the nuclear membrane until the anterior pole of the nucleus is reached; after it has been deposited on the nuclear membrane, the Golgi remnant and the other Golgi material in the tail region begin to break up into smaller bodies which ultimately become scattered through the cytoplasm. At the same time the mitochondria form a more compact body which closely surrounds the axial filament (fig. 39, Pl. 13).

The nucleus of the spermatid now comes to stain deeply, the acrosome increases in size and begins to spread out over the anterior pole of the nucleus, the mitochondria have formed a thin sheath surrounding the axial filament, and the Golgi bodies are distributed through the cytoplasm of the developing tail region (fig. 46, Pl. 13).

As these changes are taking place a clear area or vesicle appears in the posterior part of the nucleus; this vesicle is at first small, but rapidly increases in size (fig. 46, Pl. 13). In the young spermatid the chromatin becomes distributed throughout the nucleus, so that, at this stage, the nucleus is a lightly stained homogeneous body. The chromatin now appears to recede towards the central region, and a clear area, which is most marked in the posterior part of the nucleus, is formed inside the nuclear membrane and surrounding most of the chromatin. As the nucleus elongates the clear area increases in size in the posterior part of the nucleus, but is reduced in the other regions. At a slightly later stage the vesicle is a conspicuous feature of the part adjacent to the centrioles, while the chromatin is confined to the anterior and middle regions of the nucleus (figs. 35, 46, 47, and 48, Pl. 13).

The acrosome grows out to form a lightly stained structure at the anterior pole of the nucleus, and the nuclear vesicle increases in size (fig. 47, Pl. 13). Meanwhile, a comparatively large, darkly stained body has made its appearance in the region previously occupied by the centrioles (figs. 46 and 47, Pl. 13). It is suggested that this structure represents the post-nuclear body which has increased in size, so that it is no longer possible to distinguish the centrioles. In slightly older spermatids the acrosome has grown out to form a pointed structure, the nuclear vesicle and the deeply stained body have undergone a further increase in size, the tail region has lengthened considerably, and the Golgi material and mitochondrial sheath are less deeply stained than in the earlier stages (figs. 48, 49, and 50, Pl. 13). In many cases a slight swelling was observed in the posterior part of the tail region; this, in all probability, is formed by residual cytoplasm which, at a later stage, is sloughed off; no direct evidence was forthcoming on this point.

Examination of late spermatids shows that the acrosome stains deeply, so that it is not possible to differentiate it from the nucleus. The nuclear vesicle has become smaller, and the post-nuclear body is still conspicuous (fig. 51, Pl. 13). At a slightly later stage the nucleus and acrosome stain in the same manner as in the preceding stage, the nuclear vesicle is no longer present; the post-nuclear body is probably closely applied to the nucleus, and with ordinary methods of technique is indistinguishable from the latter. The thickened area at the anterior end of the axial filament is identified as a centriole (fig. 52, Pl. 13). Golgi bodies are still present in the cytoplasm, but are more slightly stained in Flemming preparations, and faintly impregnated in the Mann-Kopsch material.

The nucleus and acrosome of mature sperms are darkly stained and the tail region and mitochondrial sheath lightly stained by iron haematoxylin. Owing to the small size of the sperms further details of structure were not observed.

#### IV. DISCUSSION.

According to Lutman (8) the nucleolus of the young spermatocytes of *Platyphylax designatus* is an ovoid or

spherical body, but before the meiotic division it lengthens and becomes spindle shaped. It is smaller during the stage of synapsis, and 'at this time the body seems to be part of the spireme'; after synapsis the nucleolus appears 'as a part of the much thickened spireme thread', and is divided by a longitudinal split, which becomes more marked so that the nucleolus 'opens out as a lozenge-shaped tetrad'. When the other tetrads are formed, this body is indistinguishable from them. Kleingstedt states that the nucleolus of the male germ-cells of the Trichopteron, *Limnophilus decipiens* (7), does not give rise to chromosomes, and Pchakadge, working on *Limnophilus rhombicus* and *Anabolia sorocula* (9), states that the nucleolus is a plasmosome. The present writer found no evidence in support of the view that the nucleolus of either oocytes (5), spermatogonia or spermatocytes, contributes directly to the formation of the chromosomes of *Stenophylax*. In material treated according to Feulgen's technique, the nucleolus did not give the chromatin reaction, but was stained by light green. The nuclei in this material were 'fuzzy' and the preparations were inferior to those obtained during a study of the oogenesis of *Stenophylax*. It has been previously recorded, during an investigation on a Tenthredinid (10), that the ovaries gave better results than the testes when treated by Feulgen's method.

As previously recorded (5), the writer believes that the haploid number of chromosomes is thirty and the diploid sixty. This agrees fairly closely with Lutman's findings of fifty-five to sixty chromosomes for *Platyphylax* (8); Kleingstedt (7) claims that the female of *Limnophilus decipiens* is heterogametic with the diploid number of nineteen, and that the male has the diploid number of twenty chromosomes.

Lutman does not deal with the mitochondria or Golgi bodies, but figures elongate and rounded bodies lying beside the nucleus of some of the primary spermatocytes. As most of his material was fixed in weak Flemming, these bodies may represent imperfectly preserved clumped mitochondria. Again, in dealing with the centrosomes he states that small dark granules are present in the cytoplasm, but that it is impossible to identify

the centrosomes except when situated at the poles of the spindle; in all probability these granules are mitochondria.

The spermateleosis of *Platyphylax* is not described in detail, but Lutman states that after the axial filament appears the chromatin forms a hollow sphere inside the nuclear membrane; later 'the nuclei become very sensitive to the fixing reagent', and practically 'all of them have collapsed'.

The present findings on the Golgi material of the spermatogonia and spermatocytes of *Stenophylax* differs considerably, in certain respects, from the conditions observed in other insects. As there does not appear to be any previous investigations on the cytoplasmic inclusions of the male germ-cells of the Trichoptera, it is of interest to note the conditions in the two most nearly related groups—the Lepidoptera and the Mecoptera. Gatenby has shown that the Golgi elements of the spermatocytes of certain Lepidoptera (3) occur as curved rods, and Bowen, working on *Pygaera* and *Callosamia* (2), states that rod-shaped Golgi bodies are present. According to these authors the elements remain separate and do not clump together in either spermatogonia or spermatocytes. Poluszynski (11) has shown that ring-shaped Golgi bodies are present in the spermatocytes of *Panorpa communis*; these do not clump except to form a loose mass around the poles of the spindle.

The clumping of the Golgi material into a dense mass in the spermatogonia, and again in the spermatocytes of *Stenophylax*, appears to be a unique feature in insect spermatogenesis. The behaviour of the Golgi elements after the formation of the spindle does not call for any special notice, except that they are smaller and more numerous, and consequently are concluded to arise by division or fragmentation from the earlier elements. The amount of Golgi material present after each cell division strongly suggests that it increases at the expense of some substance in the cytoplasm. The separation, in the primary spermatocytes, of the original Golgi mass into two groups is uncommon. Hickman (6) has recently shown that the Golgi rods of the first spermatocytes of the mollusc, *Succinea ovalis*, are at first grouped round the idiosome; later the rods

move out through the cytoplasm and form two groups of Golgi elements. These groups are figured as consisting of discrete Golgi rods.

There is no doubt that in *Stenophylax* the acrosome is formed under the influence of the Golgi material. In *Pygaera* (2) acrosomic vesicles are formed as products of the Golgi bodies and are deposited on the nuclear membrane where they fuse to form the acrosome; granules which are differentiated within the vesicle also fuse to form a large acrosomal granule. Bowen states that the acrosome of *Callosamia* is so small that the early stages cannot be followed with ease; he also mentions that the acrosome tends to stain deeply, and believes that this may be due to faulty staining, as when the Golgi bodies move away the acrosome is similar to that of *Pygaera*. The small size of the acrosome of *Stenophylax*, together with the manner in which the Golgi material closely invests it, renders observations on the details of its formation impossible; its failure to show differentiation into granule and vesicle cannot be due to faulty staining, as its appearance is similar in different preparations, and as it still stains darkly long after the Golgi remnant has moved away. The subsequent behaviour of the Golgi material appears to be along the usual lines and, consequently, calls for no special comment.

It is impossible that the dense mass of Golgi material present in the spermatogonia and spermatocytes has been produced as the result of faulty technique, as a large number of both Mann-Kopsch and Flemming (without acetic) preparations were examined, and the same type of Golgi mass was observed in each case. The writer believes that this mass is not identical in structure with the classical Golgi apparatus of vertebrate material, but rather that it is to be looked upon as a substance which runs together at a certain stage in the history of the cell, and at another stage is converted into a number of separate bodies which, after cell division, come together again to form a mass similar to that present in the cell of the previous generation. The separate bodies of the male germ-cells of *Stenophylax* are, therefore, not discrete elements which exist as individual bodies throughout the greater part of the course of

spermatogenesis as in the Lepidoptera and in *Panorpa*. Consequently, in the present contribution the term Golgi material or Golgi mass is used to describe the localized condition, while the term Golgi element and Golgi body are used as a matter of convenience to describe the Golgi substance in the dispersed condition. It is of interest that in the young oocytes of *Stenophylax* (5) Golgi elements, in the form of rings and granules, are clumped at the pole of the nucleus; these do not fuse into a dense mass, and in the older oocytes have increased in number and size and have become distributed throughout the ooplasm.

There is reason to believe that the small granule which appears, at an early stage of spermateleosis, between the nucleus and the centrioles is a post-nuclear body, as at a later stage this region is occupied by a large, deeply stained body, which in the late spermatid disappears from view, apparently becoming closely applied to the posterior part of the nucleus. Gatenby and Wigoder (4) claim that granules described and figured by Bowen in the insect spermatid are, in the light of recent work, post-nuclear bodies. If this conclusion be correct it is probable that the body observed during the present investigation is of the same nature.

The significance of the clear vesicle which arises in the nucleus of the spermatid is difficult to explain. It was observed in testes fixed in the fluids of Bouin and of Flemming, and in material treated according to the method of Mann-Kopsch; its presence and its similarity of appearance in all these preparations would appear to exclude the possibility that it is an artifact. Bowen in a study of the spermatogenesis of the Hemiptera (1) has shown that the chromatin forms a thin layer on the inside of the nuclear membrane; later the chromatin draws away from the region adjacent to the centrioles, so that a transparent area is formed in the posterior part of the nucleus. Finally, the clear area becomes indistinct and disappears. Bowen also describes a clear area in the spermatids of *Pygaera* and of *Callosamia* (2), which is situated in the anterior part of the nucleus, and later disappears, during the elongation of the acrosome. The nuclear vesicle of *Stenophylax*, in its situation, and in respect to its disappearance, more closely resembles that of

the Hemiptera than that of the two Lepidoptera mentioned above. In *Stenophylax* the vesicle is obliterated by the elongation of the deeply stained mass of chromatin which spreads out to form a rod-shaped body.

The behaviour of the mitochondria does not warrant any special comment, except, it may be noted, that they do not form a definite mass or 'Nebenkern', but approach the axial filament as a collection of tangled threads, and form a compact body only after they have become arranged round the axial filament.<sup>1</sup>

#### V. SUMMARY.

1. The structure of the nuclei of the spermatogonia, spermatocytes, and the nuclear changes during the division of the spermatocytes are described. The nucleolus of the spermatocytes of *Stenophylax* does not contribute directly to the formation of the chromosomes as claimed by Lutman (8) for *Platyphylax designatus*. The diploid chromosome number of *Stenophylax stellatus* is sixty.

2. The Golgi material of the spermatogonia and of the early primary spermatocytes is in the form of a dense mass; in the primary spermatocytes this mass separates to form two masses. The Golgi material of the spermatogonia and spermatocytes breaks up to form Golgi bodies which move towards the poles of the spindle, and are thus distributed with approximate equality to the resulting cells.

<sup>1</sup> Since the preceding account was completed for publication, a paper by Sajiro Makino and Hisao Kichijo on the Trichopteron, *Stenopsyche grissipennis* ('Journ. Faculty Sci. Hokkaido Imper. Univ.', Series VI, Zool., vol. III, no. 1, 1934), has come into the hands of the present writer. Makino and Kichijo claim that the female of *Stenopsyche* is heterogametic. A nucleolus was identified in the spermatogonia, but later disappeared; it is concluded that the nucleolus has nothing to do with the chromosomes. These authors cite a recent paper by Kleingstedt ('Notulae Entomologicae', xi, 1932) who claims that the female of *Limnophilus decipiens* and of *Limnophilus lunatus* is heterogametic. Makino and Kichijo also refer to a paper by Pchakadge ('Arch. Russ. Anat. Hist. Embr.', 1930) who has investigated the chromosomes of twenty-four species of Trichoptera, but does not appear to have found that the female is heterogametic.

3. Granular mitochondria are present in the spermatogonia; during the metaphase they are distributed throughout the cytoplasm. During the division of the spermatocytes the mitochondria come together to form two masses at the side of the spindle; in the telophase these masses are separated into two approximately equal parts.

4. The acrosome is formed under the influence of some of the Golgi material; after its formation the Golgi material breaks up into smaller bodies which become distributed through the tail region.

5. The mitochondria, in the form of a tangled mass of filaments, become arranged around the axial filament; later they form a compact sheath.

6. A vesicle makes its appearance in the nucleus of the young spermatid and increases in size, but later becomes smaller and finally disappears. A structure which is probably a post-nuclear body was observed in the young spermatid.

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## DESCRIPTION OF PLATES 12 AND 13.

### LETTERING.

*A.*, acrosome; *A.F.*, axial filament; *C.*, centriole; *Ch.*, chromatin; *Cy.*, cyst-wall; *Ep.*, epithelial nucleus; *G.E.*, Golgi element; *G.M.*, Golgi material in localized condition; *M.*, mitochondria; *M.S.*, mitochondrial sheath; *N.*, nucleus; *Nu.*, nucleolus; *P.N.*, post-nuclear body; *V.*, vesicle.

### PLATE 12.

- Fig. 1.—Primary spermatogonium with epithelial nucleus. Flemming.  
 Fig. 2.—Secondary spermatogonium. Flemming.  
 Fig. 3.—Leptotene stage. Flemming.  
 Fig. 4.—Early synizesis. Tellesyniczky.  
 Fig. 5.—Synizesis. Tellesyniczky.  
 Fig. 6.—Pachytene stage. Bouin.  
 Fig. 7.—Diffuse stage. Flemming.  
 Fig. 8.—Division of primary spermatocyte; polar cap showing thirty chromosomes. Flemming.  
 Fig. 9.—Division of primary spermatocyte, showing chromosomes, centrosomes, and spindle. Flemming.  
 Fig. 10.—Division of primary spermatocyte. Telophase. Flemming.  
 Fig. 11.—Secondary spermatocyte; chromatin clumped in nucleus. Flemming.  
 Fig. 12.—Primary spermatogonium. Mann-Kopsch.  
 Fig. 13.—Primary spermatogonium; showing Golgi material breaking up into smaller bodies. Mann-Kopsch.  
 Fig. 14.—Primary spermatogonium; later phase in dispersal of Golgi material. Mann-Kopsch.  
 Fig. 15.—Primary spermatogonium; Golgi elements distributed through cytoplasm. Mann-Kopsch.  
 Fig. 16.—Primary spermatogonium; metaphase. Mann-Kopsch.  
 Fig. 17.—Primary spermatogonium, showing Golgi material and mitochondria. Flemming (without acetic).  
 Fig. 18.—Division of primary spermatogonium. Flemming (without acetic).  
 Fig. 19.—Secondary spermatogonia. Mann-Kopsch.  
 Figs. 20 and 21.—Secondary spermatogonia; anaphase and telophase. Mann-Kopsch.  
 Figs. 22-6.—Primary spermatocyte; growth stage, showing mass of Golgi material dividing to form two groups of Golgi elements. Mann-Kopsch.

Fig. 27.—Primary spermatocyte; prophase. Mann-Kopsch.

Figs. 28 and 29.—Primary spermatocyte; metaphase and telophase, showing Golgi material and mitochondria. Flemming (without acetic).

Fig. 30.—Secondary spermatocytes. Mann-Kopsch.

Figs. 31 and 32.—Secondary spermatocytes; metaphase and telophase. Flemming (without acetic).

#### PLATE 13.

Fig. 33.—Young spermatids, showing Golgi material and mitochondria. Flemming (without acetic).

Figs. 34 and 35.—Spermatids, showing mitochondria, Golgi material, centriole, post-nuclear body, and axial filament. Flemming (without acetic).

Fig. 36.—Spermatid; same stage. Mann-Kopsch.

Fig. 37.—Anterior part of spermatid, showing Golgi material at anterior pole of nucleus. Flemming (without acetic).

Fig. 38.—Spermatid; mass of Golgi material at anterior pole of nucleus; Golgi material in tail region; mitochondria around axial filament. Mann-Kopsch.

Fig. 39.—Later stage, showing mitochondrial sheath. Mann-Kopsch.

Figs. 40 and 41.—Showing acrosome at anterior pole of nucleus. Flemming (without acetic).

Fig. 42.—Showing Golgi material at anterior pole of nucleus. Mann-Kopsch.

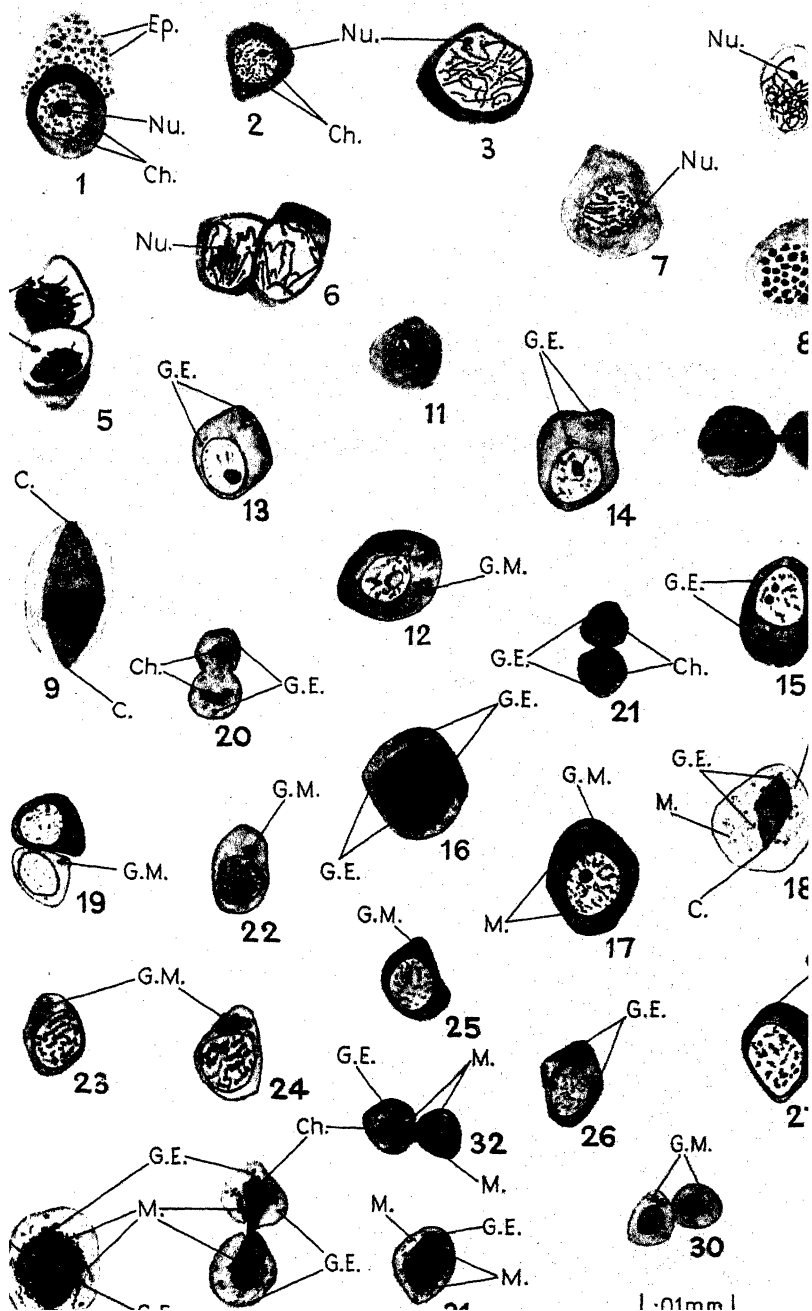
Fig. 43.—Next stage; the Golgi remnant has moved into the tail region; the acrosome is not shown; mitochondria forming sheath around axial filament. Mann-Kopsch.

Fig. 44.—Spermatogonia and cyst-wall. Flemming (without acetic).

Fig. 45.—Primary spermatocytes and cyst-wall. Flemming (without acetic).

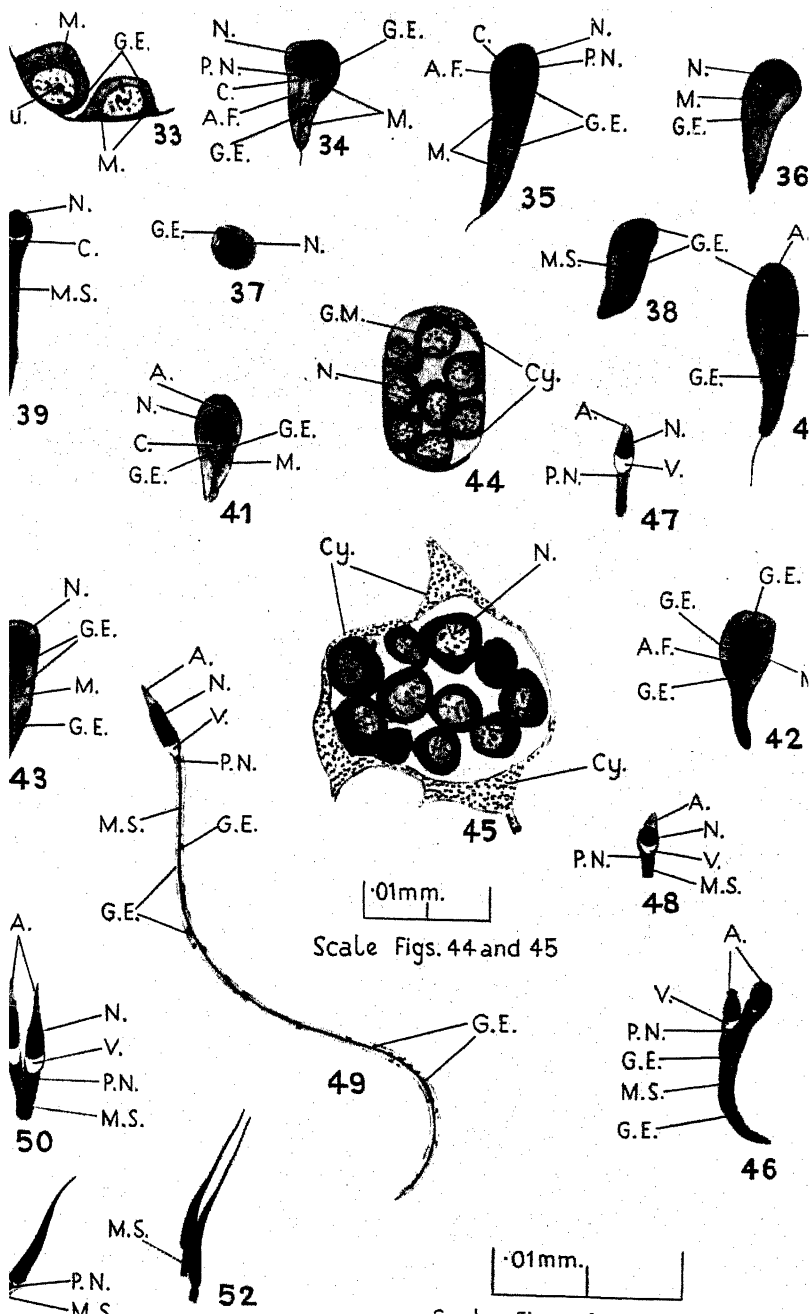
Figs. 46-52.—Late spermatids, showing development of acrosome, post-nuclear body and vesicle. Flemming (without acetic).





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# The Development of the Adrenal Gland of the Mouse.

By

H. Waring, M.Sc.

(University of Liverpool.)

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With Plates 14 to 18 and 4 Text-figures.

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## I. INTRODUCTION.

MASUI and Tamura (1926) found that in the mouse the inner zone of the adrenal cortex, called by them the reticular zone, degenerates in the male during early life, disappearing almost completely before maturity. They state that in the female this zone is retained, but undergoes fatty degeneration during pregnancy, recovering sometime after parturition. They further state that in the virgin female the zone degenerates only at senility. The origin of this zone is not clear. It is said to 'appear' between the medulla and cortex at 5 to 15 days after birth.

Miller (1927), studying the mouse adrenal, described almost the same phenomena. She describes the early degeneration of the inner zone in the male, but calls this zone the X-zone pending further investigation. The zone is described as 'appearing'



round the medulla and becoming 'definitely evident at 10-14 days'. Its more exact origin is not described. She points out a possible analogy between degeneration of this zone and that of the boundary zone of the human infant. She describes the degeneration of the X-zone in the female during pregnancy, but unlike Masui and Tamura, she finds spontaneous degeneration (both fatty and non-fatty) in the female during the first half of the reproductive period, even in the absence of pregnancy. She describes the development of a reticular zone in the female, external to the X-zone, at about the beginning of the disappearance of the X-zone.

Deansley (1928) also investigated the adrenal gland of the mouse and came to practically the same conclusion as Miller. She describes fatty and non-fatty degeneration in the virgin animal, and states that in pregnancy degeneration is more rapid but does not differ in other respects from that occurring in the unmated female. She (Deansley) describes a reticular zone in both sexes and homologizes it with the X-zone.

It is clear, therefore, that in the young adult mouse the adrenal gland shows a definite sex difference. It is equally well established that in this animal part of the adrenal cortex undergoes degeneration during pregnancy. It is by no means clear, however, exactly which part of the cortex undergoes degeneration. It is sometimes called the reticular zone, sometimes more guardedly the X-zone, but it is not even clear whether these two terms apply to the same tissue or whether two separate tissues are involved, and certainly the homology of the X-zone is an open question.

Work carried out in this department during the year 1981-2 on the adrenal gland of the cat (Davies, unpublished) has shown that in that animal there is no sex difference in the adrenal gland, such as is found in the mouse. Neither is there fatty degeneration of any part of the cortex during pregnancy. In both sexes, however, at birth there is a well-developed inner cortical zone interlocking with the medulla, and this gradually degenerates during the first few months of post natal life, ultimately disappearing entirely. A reticular zone is not recognizable in the cortex until just before the inner zone has dis-

appeared. It is practically certain therefore that these two zones are separate entities, and Davies points out that the inner zone is probably homologous with the boundary zone of the human gland described by Keene and Hewer (1927) and Cooper (1925).

Other work, carried out in this department during the past year, on the rabbit (Roaf, in the press), has shown that in this animal there is fatty infiltration of part of the adrenal cortex during pregnancy. And here it is without doubt the reticular zone which becomes infiltrated, for even in the adult both a reticular zone and a more internal zone, interlocking with the medulla, are very well defined and there can be no confusion in identification.

It does seem, therefore, that in the cat and in the rabbit the reticular zone is quite distinct from the inner zone which interlocks with the medulla. Also in the rabbit, where fatty infiltration takes place it is the reticular zone and not the interlocking zone which is involved.

In the light of these facts one questions more than ever the exact homology of the zone which undergoes fatty degeneration in the mouse.

The present investigation of the development of the adrenal gland of the mouse was undertaken, therefore, in order, if possible, to throw some light on the homology of the different zones.

I wish to express my thanks to Mrs. Bisbee (Ruth C. Bamber) for originally suggesting the work, and for her continued help and criticism throughout the course of the investigation.

## II. MATERIAL AND TECHNIQUE.

The mice used in the present investigation were kept and bred in the animal house of this department, a closed brick building artificially heated during the cold months.

Dated embryos were obtained by putting two females for one night with a male and removing them the following morning. The day they were removed was counted as the first day of pregnancy (after Inaba, 1891).

The material required for post natal development was obtained by putting several females with a male and isolating them when they became obviously pregnant. All young were weaned at 3 weeks.

Chloroform was used as the killing agent. The embryo was pressed out of the allantois and chorion, the head and thorax removed, and the abdomen opened. It was then plunged into the fixative, and when hardened the superfluous body-wall flaps were removed. The adrenals were finally sectioned *in situ*. With large post natal material only a slice of the body containing the adrenals was fixed. In these cases also the adrenals were sectioned *in situ*.

In some cases the adrenals were dissected out and fixed separately. The object in fixing the glands *in situ* was to study any nerve connexions which might exist, particularly after the sympatho-chromaffin immigration has ceased.

Most of the material was fixed in Bouin's fluid; Zenker-Formol, Muller's, and Flemming's fluids were also used, but were not so satisfactory. The majority of the sections were stained with Ehrlich's haematoxylin and eosin. In some cases Mann's methyl blue eosin was used, but not with such good results. With some sections of later stages Mallory's triple stain was used to define connective tissue. For the early stages of cell migration a chromic fixative (Ciaccio's) was used with the object of defining the sympatho-chromaffin cells by their affinity for chromic acid. This method failed in its primary object—but the general fixation was good.

All the material was embedded in 52° C. or 54° C. paraffin wax, according to season, and the blocks were sectioned at 3 $\mu$ .

Some early workers, notably Soulié (1903) and Fusari (1898), give only measurements of embryos as indications of age. This method, while giving a fair idea of the stage of development, is not so satisfactory as the use of material of definitely known age, for there appears to be a considerable variation not only among the embryos of one litter and of different litters but even more among the embryos of different strains.

Inaba gives the following figures, the embryos being measured from 'the tip of the head to the root of the tail'.

	mm.		mm.
11th day . . .	3-4.5	14th day . . .	8-10
12th ,, . . .	4.5-6	15th ,, . . .	10-12
13th ,, . . .	6-8		

I have not dealt with sufficient material to generalize on the difference between Inaba's material and mine; neither did I measure all the available material; but the difference observed is sufficiently great to be worthy of mention. The following are figures from my own stock, the measurements being taken from the tip of the head to the root of the tail.

	mm.		mm.
13th day . . .	16-17.5	16th day . . .	23-26
14th ,, . . .	18-20	17th ,, . . .	27-29
15th ,, . . .	21-22	19th ,, . . .	32-33

Thus the difference between Inaba's figures and my own is very great and the possibility suggests itself that this may be due to different methods of measurement. For instance, it is just possible that Inaba may have measured laterally from tip of head to root of tail, whereas I have measured along the mid-dorsal line from tip (crown) of head to root of tail—in which case the curvature of the embryo would be responsible for the difference in figures.

Two pregnant mice which I obtained from the Bangor colony, however, gave embryos which, when measured by my method, agree with Inaba's figures. It is clear, therefore, that the difference in size is a real one and not merely the result of an accidental difference in technique.

I have also noted that birth may occur at 19-21 days, and this would suggest that even age is not an absolute criterion of development. It would appear, however, that the age of material is a safer criterion of development than is measurement. Castle and Gregory (1929), working on large and small races of rabbits, found that within a species size difference was produced by the rate of cell multiplication being more rapid in the large race, but that differentiation is approximately equal in both races.

## III. THE DEVELOPMENT OF THE ADRENAL GLAND.

## 1. The Cortical Anlage.

## (i) Historical.

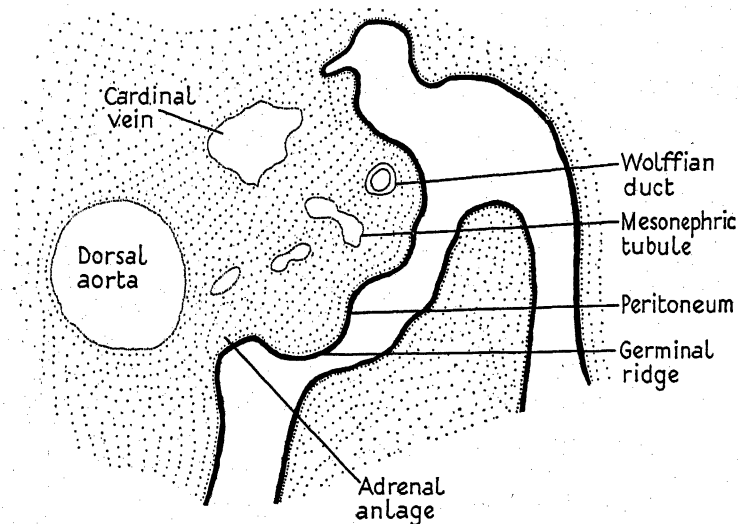
In regard to the origin of the adrenal cortex there has been considerable difference of opinion. Many workers, studying entirely different types, have concluded that it originates from indifferent mesoblast or peritoneum (Balfour, 1878; Kölliker, 1879; Mitsukuri, 1882; Rabl, 1896; Soulié, 1903; Roud, 1902; Zuckerkandl, 1912; Hays, 1914; Wieman, 1920; Pankratz, 1931). Others have claimed that it arises from some already constituted organ as, for example, from the mesoderm of the blood-vessels (Braun, 1879; Gottschau, 1883), from the pronephros (Semon, 1890; Rabl, 1891), or from the mesonephros (Weldon 1884, *a* and *b*, 1885; Semon, 1887; Hoffman, 1892). Finally, Janosik (1883), Inaba (1891), and Fusari (1893) have emphasized the close connexion of the cortical anlage with the anlage of the genital organ and, taking a more extreme view, Mihalcovics (1885) considered that the suprarenal body is only the anterior continuation of the sexual organ.

Soulé (1903) and Minot (1892), believers in the derivation of the cortical anlage from the peritoneum and general mesenchyme respectively, offer interesting criticisms of the views in regard to the origin of the anlage from definitely constituted organs.

Soulé points out from his own observations that after the anlage has arisen from the peritoneum it does make secondary connexions with (1) the glomeruli and segmental tubules of the Wolffian body; (2) the genital organ; (3) the liver; (4) the mesentery. This very fact renders suspect any theory of the origin of the anlage from any of these organs. He further points out that it is not possible to consider the suprarenal capsule as derived from the pronephros because in most mammals there does not exist a formation comparable to the pronephros, and in the mole as in the bird (where a pronephros is developed), the adrenal anlage first appears clearly at a period when the pronephros is completely formed. The distance between these two formations is too considerable for one to hope to establish the least relationship between the two. Finally, Soulié points

out that a cortical anlage in the middle of the mesoderm, as described by Braun, Kölliker, and Mitsukuri, is obviously a secondary stage.

Minot (1892) criticizes all attempts to state the exact derivation of the cortical anlage. He writes 'the recognition of the genetic relation of the whole mesenchyma to mesothelium renders it



TEXT-FIG. 1.

Diagrammatic transverse section showing position of the cortical anlage in a 12 days embryo.

unnecessary to assume a special relation for a single mesenchymal organ'.

## (ii) The Cortical Anlage of the Mouse.

The earliest adrenal enlargen which I have been able to observe were found in embryos of 12 days. At this stage, serial transverse sections show a group of cells immediately above the peritoneum in the region where the nephric tubules pass towards the Wolffian duct, and situated in the angle of the mesentary between the aorta and the germinal ridge (Text-fig. 1). Owing to the extreme twisting of the embryos at this age it was not possible to define

the position of the anlage exactly in relation to the segmentation of the embryo. The cell-mass constituting the adrenal anlage is distinguishable from the surrounding mesenchyme solely by the closer aggregation of its nuclei and its more densely crowded cytoplasmic granules. It is probably characteristic also that more mitotic figures are discernable within its limits than within the surrounding mesenchyme. The nuclei are essentially like those of the surrounding mesenchyme, being slightly ovoid and distinctly vacuolated.

The anlage of the gonad is well developed at this age, and in my preparations exactly resembles the structure described and figured by Brambell (1927, Pl. 30, figs. 1 and 2). The fact that there is no connexion whatever at this stage between the adrenal anlage and any other organ makes it extremely probable that the anlage has arisen *in situ* by proliferation from the peritoneum. By the end of the 13th day this anlage, which is destined to form the cortex of the adrenal, is consolidated and separated from the general mesenchyme by a sheath.

So far as I have been able to trace there have been only four previous workers engaged on the adrenal anlage of the mouse, namely Inaba (1891), Fusari (1893), Soulié (1903), and Roud (1902).

My own observations are entirely in harmony with those of Inaba in regard both to the structure of the anlage and to the age of its appearance, as well as in regard to its mode of origin from the peritoneum. Inaba, however, was able to define the longitudinal position of the anlage more exactly than I have done. He places it at 'about the middle of the anterior two segmental tubules to the 6th and 7th tubule'. According to his description the anlage is consolidated and separated from the peritoneum by the 13th day. On one point, however, my own observations do not agree with those of Inaba. The cells which I interpret to be the developing anlage, while occupying the identical position figured by him, have certainly not the granular nuclear appearance which he figures, but possess more vesicular nuclei as described, but not figured, by Pankratz for the rat (1931).

Fusari's evidence also supports that of Inaba in regard to the mode of origin and age of appearance of the adrenal anlage.

Soulié's observations on the mode of origin of the anlage and on the age at which it appears are also in harmony with those of Inaba, Fusari, and myself. His account of the position of the anlage is not quite clear, but, as far as one can judge, on this point also he agrees with the workers already quoted. Referring to embryos of 4-5 mm. (i.e. 11-12 days according to Inaba), he describes the epithelium which covers the mesonephros as of the same thickness all round except against 'l'extrémité super-interne' where it is formed of prismatic elements and where in some places there exist little cellular masses which can be interpreted as centres of proliferation destined to the formation of the suprarenal chords. He says that in a 7 mm. embryo (= 13th day Inaba), the adrenals are little nodules isolated in the mesoderm having lost all connexion with the peritoneal epithelium. They start to group around the neighbouring veins—on the left against the vein internal to the Wolffian body, on the right against the inferior vena cava which is in the course of formation.

Roud describes in the 14 mm. stage (= 11th day Inaba), three anlagen, genital, adrenal, and prevascular, all discernible as proliferations from the mesothelium. By the time the 7.5 mm. stage (= 13th day Inaba) has been attained the adrenal anlage has lost its connexion with the peritoneum. By the 10.5 mm. stage (= 14th day Inaba) the cortical anlage consists of large irregular cells with no cell-walls and is densely protoplasmic. At this stage and later the central vein is well developed and the protoplasm stains intensely.

There seems to be little doubt therefore in regard to the time, place, and mode of origin of the adrenal anlage in the mouse.

## 2. The Medullary Anlage.

### (i) Historical.

Practically all workers have agreed that the medullary elements come from the same tissue as the sympathetic nervous system. Balfour (1885), Rabl (1891), Inaba (1891), Soulié (1903), &c., &c.

So far as I can trace, only three authors have taken the view that the adult medulla is cortical in origin. 'Janosik and Gottschau do not dispute the statement that nerve-fibres enter



the anlage, but they state that the two parts of the adrenal substance cannot be distinguished at the time of entrance, and that the medullary substance is differentiated at a later stage' (statement from Inaba). Minot takes a similar stand up to 1892. In 1886 he says, 'the medullary cells are unlike the cortical but earlier the cortical cells have the same characters as the medullary being large and irregular in size' (p. 312), and again in 1892 he writes, 'that both the cortex and medulla of the adult organ are formed in man from the mesenchymal cells, as Gottschau showed was the case in several mammals, is I think, beyond question. Gottschau showed that the medulla did not arise from the sympathetic anlage, but he failed to ascertain what became of the sympathetic masses. I have ascertained that there are groups of cells which gradually disappear, and in appearance they resemble the cells consigned to a sympathetic origin in the rabbit' (p. 487).

All other workers agree that the medulla arises from the invading sympathetic elements, and it will be seen that my own observations completely fall in line with this view. Nevertheless, one can well understand how there could be confusion in regard to the identity of the different adrenal elements in the early post natal stages of the mouse, for the sympathetic cells change their size and shape and become more like the cortical cells. These changes are discussed below (p. 348, cf. p. 353).

The generally accepted account of the development of the adrenal gland is that outside the cortical anlage, closely adpressed to it, a sympathetic nervous mass arises. Some workers (Keene and Hewer, 1927; and Inaba, 1891) have described its development from the sympathetic nervous system. From this associated sympathetic mass, cells migrate into the cortex and eventually form the medulla. There is a certain amount of laxity in the terminology. The cells which migrate into the cortical anlage are sometimes referred to as the sympathochromaffin cells because of their affinity for the salts of chromium and from the fact that they clearly migrate from a sympathetic ganglion. It is assumed by most writers that the migrating cells are truly nervous in nature (Keene and Hewer call them neuroblasts), and that they later metamorphose into medullary

cells. Soulié, however, takes the stand that these cells cannot be nervous in nature and refers to them as parasympathetic cells. Throughout the present paper the cells which migrate into the cortical anlage will be termed sympatho-chromaffin cells.

(ii) The Medullary Anlage of the Mouse.

On the 13th day of foetal life there is a definite sympathetic mass closely applied to the cortical anlage. This nervous mass is called by Inaba the 'splanchnic plexus'. From it sympatho-chromaffin elements will eventually migrate into the cortical anlage to form the medulla of the adult adrenal. This sympathetic mass is very quickly developed, for in sections of 12-day embryos I can find no trace of it, yet at 13 days it is well established. Inaba (1891, p. 223) gives a careful description of the origin of the splanchnic plexus from the sympathetic ganglion, but he does not give any definite age for the beginning of its formation. However, he does not figure it at all in the 12-days stage, and this concurs with my own observations. My own material gives no clue to the origin of the sympathetic mass.

3. The Union of the Anlagen to form the  
Adrenal Gland.

According to my own observations, by the 14th day the cortical anlage is clearly separated from the general mesenchyme by a well-marked mesodermal sheath. Soulié made a similar observation in 9 mm. embryos (i.e. the 14th day according to Inaba). Quite apart from its sheath, however, the anlage is clearly distinguished at this stage from all the neighbouring tissue by the densely packed state of its large finely granular cells. Inaba figures cell boundaries, but I have been unable to find any, the appearance of cells in my preparations being apparently due to a greater concentration of protoplasm round the nuclei. The cytoplasm at this stage is strongly eosinophil. The nuclei are large and sausage shaped. They have one or two eosinophil nucleoli and occasional large basiphil granules; in general, however, they might be described as clear and

vesicular (fig. 1, Pl. 14). The nucleoli show the distinctive feature of appearing bright red when stained in Ehrlich's haematoxylin and eosin, and this distinguishes them from the invading sympatho-chromaffin elements, described below, in which the nucleoli, although they take the red stain, take it so intensely that they are practically opaque and do not show the red colour except under intense illumination. In preparations of glands of this stage the blood sinus is of varying size; in the one figured it is very obvious.

It is at this stage, 14 days, that infiltration of the sympatho-chromaffin cells occurs according to both my own observations and those of Soulié and Inaba. The sympathetic nervous mass (splanchnic plexus) is now seen to be incompletely divided into two parts by connective tissue (fig. 1, Pl. 14, cf. Fig. 12, Pl. 18). The actual cells appear to be of two kinds. Firstly, there are the large ganglion cells with large clear round nuclei, each with one or more large eosinophil nucleoli, and a few coarse basophil granules. Their cytoplasm is finely granular, almost as fine as the cytoplasm of the future cortical cells. They have the typical appearance of ganglion cells. There are also small nuclei, not clearly associated with cytoplasm, which stain very intensely with haematoxylin. According to my preparations it is these latter cells which penetrate into the cortical anlage.

I have been able to observe two kinds of infiltration, one on the late 14th, the other on the 15th day of embryonic life.

The first type observed (fig. 1, Pl. 14) is that in which individual deeply staining sympatho-chromaffin elements, unconnected with any nerve-fibres, migrate from the closely applied sympathetic mass into the spaces of the cortical anlage. Nerve-fibres can be seen within the organ and entering its substance at different points but unaccompanied by sympatho-chromaffin cells.<sup>1</sup>

The second type (fig. 2, Pl. 15) is that described by Inaba (1891) for the mouse and by Pankratz (1931) for the rat. Fibres enter the cortical anlage from the sympathetic mass closely applied to it. The fibres run deeply into the cortical substance

<sup>1</sup> As the innervation of the gland is not part of the main study it is not discussed in this paper.

and along their length, and at their cortical extremities there are small sympatho-chromaffin cells.

Roud also describes the migration of the future medullary cells along the track of nerve-fibres, entering the cortical anlage; but an essential point of difference lies in his description of the entrance of these nerve-fibres into the cortical anlage at the 8.5 mm. stage (= 14th day Inaba), while he does not describe a definite sympathetic mass applied to the adrenal until the 10.5 mm. stage (= 15 days).

According to my own observation the sympatho-chromaffin cells, once within the cortical anlage, form nests in the inter-cellular spaces and clearly correspond to the 'rosettes' of chromaffin cells described by Pankratz in the rat, and by Keene and Hewer in man.

Whether there be two types of migration or whether I have observed only two different phases of the same type is not certain.

There also seems to be some doubt from the records of other workers as to how many types of cells migrate from the sympathetic mass into the cortical anlage. I myself have seen only one type migrate, namely the small dark granular nuclei with their uncertain quantity of cytoplasm. I have been unable to obtain any evidence of immigration of the ganglion cells. Inaba (1891) also describes only one kind of migrating cell in the mouse and my observations are entirely in harmony with his findings in this respect. Keene and Hewer (1927) describe two types of migratory cells in the human gland, quite apart from the ganglion cells. 'The most numerous of the two types of cells are small with darkly staining nucleus and very little cytoplasm; possibly these are neuroblasts because they are frequently found with nerve-fibres . . . the second type of cell is larger than the neuroblast and has a vesicular nucleus.' The former of these cell types obviously corresponds with those shown in my own preparations; the latter type I have not identified in the mouse. Some sections, however, show cells within the splanchnic plexus intermediate, both in size and character, between the small darkly staining type and the obvious ganglion cells (fig. 2, Pl. 15). These intermediate cells

may correspond to the larger migratory type described by Keene and Hewer (1927), but there is no sign of their migration in the mouse. Unfortunately the two types of cells cannot be clearly identified in the figure given by Keene and Hewer. Pankratz (1931) also refers to the same kinds of cells in the rat, and claims to figure them, but the drawings are not annotated in the published plates and the two types of cells are certainly not recognizable. He does not discuss the fate of the cells. Pankratz also reports the immigration into the cortical anlage of a few ganglion cells.

The ganglion cells which have been described in the developing medulla of some forms, such as the rat and the human embryos, have not been described from the gland of the mouse. Neither Inaba, Soulié, nor Roud describe or figure ganglion cells in any part of their account. My own preparations show no definite ganglion cells. Pankratz describes ganglion cells in the medulla of the rat and states that they do migrate from the sympathetic mass into the adrenal anlage. He figures such cells on the migratory path. Both Keene and Hewer (1927) and Cooper (1925) describe and figure ganglion cells in the developing adrenal of man, but none of these latter workers describes the migration of these cells into the gland. Cooper (1925) accounts for their presence by metamorphosis *in situ* from sympatho-chromaffin cells. She states that in some parts of the gland the migrated sympatho-chromaffin cells 'instead of becoming chromaffin or medullary cells have developed in the opposite direction into sympathetic ganglion cells'.

A point of interest in regard to the migratory cells, which arises from the present investigation, is the change which takes place in them in the ratio of nucleus and cytoplasm. Inaba simply describes his migratory sympatho-chromaffin cells as having densely granular nuclei and less protoplasm than the cortical cells. Soulié gives no account of them. From my own preparations examined under a 2 mm. objective, the migratory cells seem to change their character immediately they are within the cortical anlage. Within the sympathetic mass and on the migratory path the small dark nuclei have an almost negligible amount of cytoplasm directly associated with them or

concentrated round them, except in rare cases where there is a 'tail-like' projection (see fig. 1, Pl. 14). Where they have penetrated deeply into the cortical mass, however, the nuclei, which have undergone no change, have associated with them a considerable quantity of basophil cytoplasm which contrasts with the eosinophil cytoplasm of the cortical cells. I have not been able to find any reference in the literature to this change of character in the migratory cells.

Thus the early development of the mouse adrenal seems to be as follows:

A cortical anlage is formed from the mesoderm at about the 12th day.

A sympathetic nervous mass in close proximity to the developing anlage is present at about the 13th day. The sympatho-chromaffin elements migrate from the closely applied sympathetic mass into the substance of the anlage, beginning on the 14th day. By the end of the 14th day a considerable amount of immigration has taken place.

#### 4. The Development of the United Anlagen.

The further development of the adrenal gland involves the metamorphosis of the migrated cells to form the medulla; the concentration of these cells in the centre of the gland; the differentiation of the cortical cells giving the different zones of the adult cortex, and the degeneration of certain elements of the cortex lying immediately outside the medulla. The development is the same in both sexes up to 18 days after birth. From that point certain sexual differences occur.

Towards the end of the 14th day, the adrenal gland is well established. It is a small, compact, almost spherical organ flattened against the anterior end of the metanephros. It is easily seen with the naked eye and can be dissected out. Seen in transverse section, the gland consists of a mass of cortical cells with groups of sympatho-chromaffin cells scattered amongst them. These sympatho-chromaffin cells are found both isolated and in groups like the rosettes described by Pankratz (1931) in the rat. They are scattered quite irregularly throughout the gland.

The cortical cells have large clear sausage-shaped nuclei, with one or two large eosinophil nucleoli and with a few coarse basiphil granules on the inner side of the nuclear membrane. Their cytoplasm is finely granular and highly eosinophil showing a concentration round the nuclei, but with no visible cell boundaries.

The sympatho-chromaffin cells, on the other hand, consist of small ovoid nuclei which are strongly basiphil and highly granular with one or more dark nucleoli. These nucleoli, as described above (p. 340), look opaque, but on intense illumination show red coloration. The cytoplasm is coarsely granular and basiphil.

In some sections a large blood sinus is evident (fig. 1, Pl. 14) together with numerous small ones between the limbs of the cortical tissue. These blood spaces contain embryonic blood-cells and the small dark sympatho-chromaffin cells already described.

The 14-day stage is roughly equivalent to the earliest stage described by Davies (unpublished) from preparations of embryo cats. She describes a condition with sympatho-chromaffin cells scattered in the substance of the cortical anlage, representing a condition prior to their concentration in the centre of the gland as the medulla. Davies noted at this stage that the peripheral cells of the cortex are small and differentiated from the gland as a whole forming a clearly marked peripheral zone. A similar differentiation of the foetal cortex is described for man (Keene and Hewer, 1927). After a most careful examination of my own material, however, I can find no trace of such peripheral zone in the mouse.

The whole gland at this stage is surrounded by a capsule. On the peritoneal side of the gland this is of deeply staining cells and is almost fibrous, while on the aortic side the capsule is of elongated connective tissue cells, and is broken only where the sympatho-chromaffin cells enter the anlage.

### 15-day Embryos.

After 15 days of intra-uterine life there is little change except that the medullary tissue seems more concentrated at the centre

of the gland. Immigration of sympatho-chromaffin cells is still going on, and multiplication of the medullary cells is also occurring in situ. (See fig. 3, Pl. 15, showing mitoses in the future medulla.)

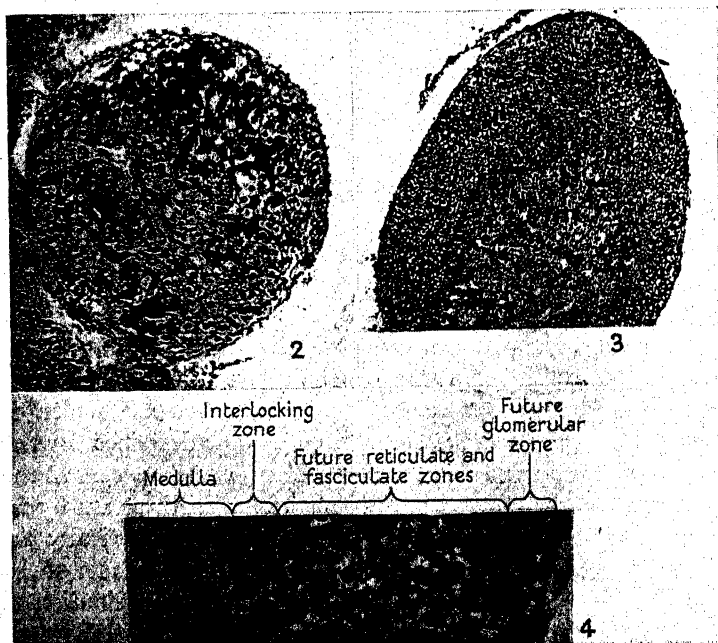
### 16 Days to Birth.

In 16-day fetuses the gland has enlarged and projects appreciably into the coelom, and possesses a well-marked capsule of similar structure to that described for the 14-day stage. During the period of 16 days to birth there is a further general increase in size and a gradual concentration of the sympatho-chromaffin elements in the middle of the gland.

The cortical cells begin to show differentiation, the majority developing a cytoplasmic content which is distinctly less dense and less eosinophil than that of the cortical mass at 14 days. The protoplasmic arrangement in this less dense region is beginning to show the vacuolate appearance described for the post natal zona fasciculata (p. 349). Moreover, these cells at the same time assume definite cell-walls, although these are by no means distinct in certain places. Some of the cortical cells, however, retain the closely packed eosinophil cytoplasm of the earlier stages and still show no cell-walls. The distribution of these more highly eosinophil cells is indefinite, but they are largely intermingled with and concentrated round the sympatho-chromaffin tissue (fig. 4, Pl. 16, and Text-fig. 2). This darkly staining non-vacuolated cortical tissue will henceforth be referred to as the interlocking zone. Towards the close of this period there is a most distinct irregularity in the arrangement of the cells at the periphery of the cortex, and this irregularity has developed parallel with an obvious tendency to crowding suggestive of rapid division. This outermost zone, although not yet fully differentiated is, by its position and characteristics, undoubtedly the forerunner of the zona glomerulosa of the adult gland. Immediately internal to this zone the cells begin to show an indistinct radial arrangement suggestive of the fasciculate arrangement in the adult gland. The nuclei of all the cortical elements are still very much alike, and during this period they show a tendency to become spherical. Though not so obviously



vacuolated as in previous stages they are still characterized by a large central cavity surrounded by somewhat coarse granules,



TEXT-FIGS. 2-4.

Fig. 2.—Photomicrograph of a transverse section of the adrenal gland of a late 17-days embryo to show differentiation in the cortical tissue. Bouin's fluid: Ehrlich's haematoxylin and eosin.  $\times 275$ .

Fig. 3.—Photomicrograph of a transverse section of an adrenal gland at day of birth. Bouin's fluid: Iron haematoxylin and Acid Fuchsin.  $\times 163$ .

Fig. 4.—Photomicrograph of part of a transverse section of an adrenal gland at day of birth. Bouin's fluid: Iron haematoxylin and Acid Fuchsin.  $\times 423$ .

and the brightly stained red nucleolus, though somewhat less bright than in previous stages, is still very distinct.

The sympatho-chromaffin cells can be distinguished as in previous stages by their small highly granular nuclei and their coarsely granular cytoplasm which stains deeply with haema-

toxylin. During this period little change is observable in their structure. They become more concentrated in the centre of the gland, however, and where cortical cells persist centrally they form strands traversing the future medullary mass. It is interesting to note that in all the sections I have examined these penetrating strands of cortical tissue are composed entirely of the more highly eosinophil type of cell.

### Day of Birth.

By the day of birth the gland has enlarged considerably. There is a close interlocking of cortex and sympatho-chromaffin tissue (fig. 5, Pl. 16; Text-figs. 3 and 4), in fact some cortical strands continue right across the central mass.

The interlocking zone of the cortex where it lies just outside the medulla is about one or two cells thick in most places but is locally thickened (fig. 5, Pl. 16, and Text-fig. 4). It is characterized by highly eosinophil cytoplasm, approximating to the condition which is found throughout the cortical substance of the 14th-day stage, and in the more highly eosinophil cells described from the cortex of embryos of 16 days and older. There may be cell boundaries visible (Text-fig. 4) but frequently none are clearly discernible (fig. 5, Pl. 16).

The rest of the cortex consists of small cells with a distinct radial arrangement, and discernible for the first time are blood-vessels running radially from the periphery towards the centre of the gland. They tend to separate the cortical cells into radial rows. The cytoplasm of this future fasciculate and glomerular region is distinctly eosinophil but not so much so as that of the interlocking zone. The cell boundaries are ill-defined, but in some places they become very distinct (fig. 5, Pl. 16, and Text-fig. 4). The condition of the future zona glomerulosa is the same as that described for embryos of nearly full term; the zone being indicated merely by the irregular arrangement of its cells as compared with the arrangement in the future fasciculate region.

The nuclei of all the cortical cells retain the same general structure as that described in detail for the 14th day of foetal

life; that is to say, they are vesicular with a few coarse basophil granules and an eosinophil nucleolus. The previously noted tendency to change from sausage shape to roughly spherical (see p. 345) has persisted, and there has been a general reduction in size, the nuclei of the interlocking zone, however, becoming on an average slightly smaller than those of the future glomerular and fasciculate zones. In the region of the future zona glomerulosa the nuclei are more closely packed than elsewhere. In fact in the whole cortex at this period, but in this zone in particular, the small amount of protoplasm associated with each nucleus suggests active division, although mitotic figures are rare.

The sympatho-chromaffin elements have not as yet attained any clear cellular arrangement being, as in the previous stage, an association of nuclei surrounded by coarsely granular cytoplasm with no evident cell-walls. Both nuclei and cytoplasm are basiphil. The nuclei are of varying shape, some definitely rounded, others slightly elongate. As in the previous stage they are still deeply granular and possess two darkly stained nucleoli, but two changes have occurred which make them more like the true medullary nuclei of the adult. (From this point the cells will be referred to as the medullary cells.) The first change is that they are distinctly less granular than in the earlier stages; the second, possibly correlated with the first change, is that they have increased in size considerably. In fact the nuclei of these cells, which were in the first place the smallest in the gland, have increased to such an extent that they are now about the same size as the nuclei of the interlocking cortical zone. The characteristic deeply stained almost black nucleoli of the medullary cells are, however, in sharp contrast to the brightly eosinophil nucleoli of the cortical cells. This distinction, as well as differences in the relative granulation and vacuolation of the two types of nuclei make them very different in appearance despite their similarity in size.

Roud (1902) described and figured a zona reticularis for late stages of intra-uterine life. I have seen such a condition as he figures in material fixed with Müller's fluid, but only in imperfectly fixed material.

## Period 2-8 Days after Birth.

During this period there is a gradual change in the whole gland towards the early mature condition. The general arrangement of its cell components remains constant, with the medulla central, the inner darkly staining eosinophil zone interlocking with it, and the future fascicular and glomerular zones peripheral to it. Towards the end of this period there is a noticeable increase in the proportion of medulla to cortex and, at the same time, the capsule surrounding the gland becomes more obviously fibrous.

The interlocking zone retains the characters described above (p. 347), and during this period it becomes fairly constantly two or three cells wide. The single-celled strands completely traversing the medulla are eliminated by 8 days, and in consequence the intermingling of these tissues is not so evident as previously. The fasciculate zone shows a general increase in the size and number of cells. The cytoplasm in these cells is now concentrated round the cell boundary, and in the interior of the cell it presents a characteristic appearance—the granules are arranged in groups or clumps, such clumps being placed at fairly constant distances apart, so that the spaces are regular and from their shape might be interpreted as vacuoles. This structure remains fairly constant right through development up to the mature condition. The figure of the fasciculate zone of a 30-day old male (fig. 7, Pl. 17) illustrates the structure described. Throughout this period there is an increasing tendency for the fasciculate columns to be separated by blood-vessels so that a radial arrangement of the columns is emphasized although there is no radial elongation of the cells themselves. Towards the periphery the radially arranged columns give place to a more indefinite arrangement of cells, and at the extreme periphery the nuclei are becoming even more densely crowded than in the stage described above. It is fairly clear from the subsequent development that this region of indefinitely arranged cells, with its outer layer of densely crowded nuclei, will eventually form the adult zona glomerulosa. As the glomerular structure is most clearly defined at the extreme periphery it would appear that differentiation, of this zone at least, is

proceeding from without inwards. At the extreme periphery the crowding of the nuclei is eventually such that the cell-walls hardly seem to delimit the cells. This crowded arrangement of the nuclei of the future glomerular zone, becoming gradually more intense, despite the general increase in the diameter of the whole gland, points most strongly to this region as being a zone of rapid division, and yet the number of mitotic figures observable is surprisingly small.

All the cortical nuclei retain the structure described for the day of birth, except that those in the interlocking zone become distinctly less vacuolated. The nuclei of the fasciculate zone still remain somewhat larger than those of the interlocking zone, as in previous stages, but the difference in size is gradually becoming less marked. The more uniform size of the nuclei in these two zones appears to be due more to the reduction in the size of the nuclei of the fasciculate zone than to any increase in the diameter of the nuclei of the interlocking zone. Throughout this period, the nuclei of the peripheral and fasciculate zones are of about the same size, but in some places the former shows groups of distinctly smaller nuclei. Darkly staining nuclear bodies are present, scattered throughout the cortex—sometimes more concentrated in one place—sometimes in another. These bodies are very opaque, but under intense illumination they appear to show nuclear figures.

During the first 2 or 3 days after birth, the medulla is in much the same state as previously described (p. 348). At about 5 days, however, it has attained a definite cellular structure with cell boundaries. These cell boundaries are ill defined and delimit coarse grained basophil cytoplasm arranged in irregular strands round the nuclei. The nuclei are now considerably enlarged, and appear slightly larger than those of the cortical interlocking zone. Moreover, they become more vesicular than in previous stages, and this tendency to vacuolization persists until, by the 8th day, a distinct vacuole is manifest which henceforth distinguishes these nuclei from their unmodified sympathochromaffin predecessors. During this period the medulla shows a large number of darkly staining bodies similar to those described above from the cortex. Inaba (1891) does not figure

them either in the cortex or medulla, but Whitehead (1933, b) refers to abundant mitoses in the whole embryonic adrenal.

#### Period 9-25 Days after Birth.

The most important changes during this period involve the progressive withdrawal of the interlocking tissue from the medulla and its gradual concentration into a zone surrounding and still interlocking with the medulla; the considerable enlargement of this zone during the period 18 to 25 days; and the beginning of a sex difference in its dimensions towards the end of the period.

Whilst the interlocking tissue becomes less and less intermingled with the medulla and progressively more confined to a definite interlocking zone around the medulla, the zone itself does not appreciably increase in width for some time. At 18 days it is still only about 4 cells wide. By 21 days, however, the zone has increased considerably and a slight sex difference is discernible in its width. Counts of nuclei at this stage give an average of 8 nuclei deep for the male and 10 deep for the female. By this time, too, sections show isolated parts of this eosinophil tissue left stranded within the medulla. These isolated portions of the tissue retain the nuclear and cytoplasmic properties of the interlocking zone. In the interlocking zone itself, by this time irregular spaces have appeared running along the radii of the gland and diverging, in places, to the left and right, thus giving a vague impression of a reticular zone. These spaces are less obvious in the female than in the male. At 25 days the interlocking zone in the male has not increased beyond the dimensions recorded for 21 days, but in the female it has increased considerably. In both male and female the nuclei of this zone are closely grouped, in fact, so closely that no cell boundaries can be seen between them and, in many cases, the nuclei appear to be in contact with each other.

During this period the future fasciculate zone undergoes a gradual change towards the mature condition with cells of distinctive character elongated radially and arranged in columns which are separated by blood-vessels. Although the columnar arrangement was foreshadowed very early in development (see

p. 345) it is not until about 14-18 days after birth that it is clear that the individual cells are elongating in a radial direction. Even at this age a definite columnar arrangement is only shown locally. Sections taken at 21 and 25 days, however, show the radial arrangement very pronounced with the cells elongating radially and the columns separated by blood-vessels. The cytoplasmic condition of this fasciculate area is striking. The granules are larger than those of the interlocking zone, less densely stained and arranged in a definite manner. This arrangement appeared at a much earlier stage and was described above (p. 349), but from about 18 days on it becomes more marked. The granules are in small groups which, abutting on each other, give the appearance of a reticular arrangement with small vacuoles (fig. 6, Pl. 16). (This arrangement is even better developed by 30 days, cf. fig. 7, Pl. 17.) There is, too, a slight concentration of cytoplasm round the cell boundaries which, throughout this zone, are now very well developed. Also, at 18 days the innermost cells of the fasciculate zone, while retaining this same protoplasmic structure, begin to show a somewhat more compact reticulum and the granules develop a greater affinity for eosin.

By far the most interesting feature during this period, however, is the appearance at 25 days of a reticular arrangement of the cells at the inner ends of the fasciculate columns where they abut on to the interlocking zone. This new region is, from its future history, undoubtedly the beginning of the true reticular zone of the adult cortex. That it is being differentiated from the fasciculate region and not from the interlocking zone is clear because the cells have the same vacuolated cytoplasmic structure and definite cell-walls as the fasciculate cells and are in striking contrast to the densely cytoplasmic elements of the interlocking zone which at this stage may show no distinct cell-walls. (Fig. 7, Pl. 17, shows the reticular zone clearly established in a 30-days old male.)

The region of irregularly arranged cells peripheral to the fasciculate columns shows no change until about 18 days after birth, when it becomes much more clearly evident. The cell boundaries are indistinct and seem scarcely to limit the cyto-

plasm round the nuclei. In this area there are aggregations of black bodies strongly reminiscent of those recorded from the medulla and cortex, between birth and 8 days. Under strong illumination they show some structure, which strongly suggests that they are condensed mitotic figures (cf. p. 350).

At the extreme periphery there is that close aggregation of nuclei which has been apparent from a short time after birth, but no definite advance towards the arched arrangement of cells, typical of the adult glomerular zone, is evident until about 21 days. At that time, however, the zone does show definite signs of this arched arrangement allied to a close crowding of the cells suggestive of rapid multiplication. And further, the glomerular nature is spreading inwards involving the indefinitely arranged cells which have hitherto been present in the region immediately external to the fasciculate columns. This encroachment persists, so that at 25 days after birth there appears only a small zone of indefinitely arranged cells which is slowly giving place to the extension of the glomerular zone.

Throughout this period the cortical nuclei show little change in structure from that described for the first week after birth. Some sections of later stages, however, show an increase in the number of nucleoli, but as in the very earliest stages, one seems to be predominantly large. The increase in width of the interlocking zone, together with the crowding of its nuclei, suggests that nuclear division is taking place *in situ*, and this interpretation is supported by Whitehead's (1932 and 1933, b) analysis of cortical mitosis.

The medullary cells are enlarging during the whole of this period and continue to enlarge even after 25 days. By 14 days it is for the first time evident that the medullary cells are arranged in groups bounded by connective tissue, and this formation persists throughout life. (Treatment with Mallory's triple stain shows the cell-bundles clearly outlined in blue.) Throughout these changes the medullary cytoplasm retains the stranded coarsely granular structure previously described. The nuclei, which by 5 days had become slightly larger than those of the interlocking zone, have continued to increase in size and by 18 days they are distinctly larger than any nuclei in the



cortex (fig. 6, Pl. 16). Beyond this age there is a further but not so noticeable increase. With increasing size the nuclei become very distinctly vacuolate with one or more nucleoli and with coarse granules of nucleoplasm just within the nuclear membrane. The nucleoli also become progressively less deeply staining so that, by about 21 days, the medullary nuclei have come to be very like those of the original cortical anlage. Thus ends a remarkable series of changes from a small, highly granular, basiphil sympatho-chromaffin nucleus with dark, almost opaque nucleoli to a large, vacuolate nucleus with a large nucleolus which stains very brightly with eosin. In the meantime the cortical nuclei have undergone slight change inasmuch as the nucleoli have become progressively more deeply staining, so that by about 18 days they appear very dark indeed.

In spite of these changes no confusion of medullary and cortical nuclei is possible because throughout this period the former have been distinctly larger than the latter. Further, the two types of tissue—medullary and cortical—are by now well segregated and the basiphil cytoplasm of the central medullary mass contrasts strongly with the eosinophil cortical cytoplasm surrounding it.

By 25 days sections show some medullary nuclei large and vesicular as described previously and some slightly smaller and more granular, but I have no clue to the significance of this difference.

#### 25-30 Days after Birth.

During this period the interlocking zone retains its general structure with nuclei approximately radially arranged, surrounded by highly eosinophil cytoplasm often with no visible cell-walls (p. 351). More spaces arise, however, giving more and more a reticular appearance, particularly in view of the fact that many of the spaces are filled with blood-cells. The zone interlocks with the medulla as before, but an important change is taking place in the parts isolated within the medulla. These isolated parts seem to be considerably reduced. If this is true it is clear that either they are being pushed out or are degenerating. I have no direct evidence for either view. By 30 days there

is a most pronounced sexual difference in regard to the interlocking zone. The dimensions of the zone have steadily increased in the female, while in the male the greatest size was attained at about 21 days and from then until 30 days it has remained constant. At 30 days the beginning of a decided change is discernible in the male. At 28 days there is no sign of degeneration of the interlocking zone which lies external to the medulla; but by 30 days there is an irregular circular split running round the gland parallel to the outer circumference and situated between the interlocking zone and the reticular portion of the fasciculate zone (see fig. 7, Pl. 17). In this split at 30 days there is just one line of flattened small nuclei, staining with haematoxylin, and linked together by a strand of eosinophil cytoplasm. From subsequent behaviour it is clear that this represents the beginning of the degeneration of the interlocking zone of the male.

The previously noted reticular nature of the bases of the fasciculate columns (see p. 352), where they abut on the interlocking zone, becomes clearer in the male, but remains the same in the female as at 25 days. Close examination shows little cytological difference between the cells of this zona reticularis and those of the zona fasciculata (see p. 352). The arrangement of the cells of the fasciculate zone has become more definitely columnar and there are well-marked blood-vessels between the radially arranged columns. The condition of the cytoplasm and its staining peculiarities were described for 18 days after birth (p. 352) and since then there has been no change other than the arrangement becoming more definite.

The intermediate zone of irregularly arranged cells, between the fasciculate columns and the zona glomerulosa, is gradually becoming less extensive; in fact, in places the columns of the fasciculate zone abut directly on to the arches of the glomerular zone which is now distinctly attaining its mature condition.

The medullary cells are aggregated into bundles as described for the previous stage (p. 353). And the bundle seems to be the medullary unit, because the cell boundaries have become so ill defined that often they cannot be traced. The nuclei of the two types described at the 25-day stage (p. 354) are recognizable

at this stage within one and the same connective tissue sheath. Blood sinuses and blood-cells are plentiful.

### 30-35 Days after Birth.

The interlocking zone shows a further increase in size in the female and shows a most distinct reticular structure. In the male this zone shows no increase in size and the degeneration, noted at the beginning of the 30th day (p. 355), becomes more marked in some places but has not advanced very far in general. The nuclei of this zone in the male show a tendency to increased vacuolization.

In all other structures the two sexes remain very similar. There is a still more obvious reticular arrangement in the region between the interlocking zone and the fasciculate columns in the male—but the female has remained the same as at 25 days after birth. The fasciculate and glomerular structures remain the same in both sexes and have really attained the mature condition.

The medulla has changed very little. It is still possible to recognize stranded portions of the interlocked tissue unconnected with the continuous layer. Of the two kinds of nuclei found in the medulla—the granular and the vesicular—the vesicular seem to have reached the extreme limit of vacuolization, scarcely any nucleoplasm being visible. Blood sinuses are abundant in the medulla throughout this stage.

The subsequent development of the male and female adrenal glands will be treated separately. As a preliminary to the further history of the glands, a resumé of the events common to both sexes is given below.

### Resumé of the Development of the Adrenal Gland up to the End of the 35th Day after Birth.

A cortical anlage arises at about the 12th day of embryonic life and is constituted into a compact structure by the 13th day. Its cells have large sausage-shaped nuclei, whose nucleoli stain bright red with Ehrlich's haematoxylin and eosin. The cytoplasm is not split up by cell boundaries and is finely granular and intensely eosinophil.

The medullary anlage arises about the 13th day and consists of a mass of sympatho-chromaffin cells and nerve ganglion cells, closely applied to the cortical anlage.

Sympatho-chromaffin cells migrate into the cortical anlage on the 14th day and become arranged in groups, irregularly scattered throughout the cortical mass. The sympatho-chromaffin cells are strongly basophil and have dark almost opaque nucleoli.

Between the 16th and 18th days of embryonic development there is a differentiation of the cortical tissue. The majority of the cells become much less eosinophil, but the cells concentrated round the future medullary elements retain the deeply eosinophil character of the original cortical cells.

Just prior to birth the cortex shows signs of fasciculate columns, and at the extreme periphery there are irregularly arranged cells which foreshadow the adult zona glomerulosa.

At birth the sympatho-chromaffin cells are concentrated almost entirely in the centre of the gland. The highly eosinophil cortical cells form a zone surrounding the future medulla and closely interlocking with the medullary tissue. These eosinophil cells seem to be exactly the same as the undifferentiated cortical tissue noted above, except that cell boundaries are sometimes visible.

By 15 days after birth the inner eosinophil interlocking zone is very definite. The sympatho-chromaffin cells have acquired cell-walls and their nuclei have become distinctly vesicular.

By 21 days after birth the interlocking zone has reached its maximum development in the male and from then to about 30 days it remains constant. In the female it continues to increase throughout this period and beyond, not reaching its maximum until 35 days. The cell boundaries of this zone are inconstant in their appearance.

At 25 days after birth there is an irregularity at the base of the fasciculate columns in both male and female. In the male this becomes more definite and by about 30 days has formed an unmistakable reticular zone. In the female the arrangement remains unaltered at 30 days. Even at 35 days there is still no change, and beyond this point the history has not been followed.

## 35-42 Days after Birth in the Male.

The chief interest at this stage naturally centres on the condition of the interlocking zone. At 30 days after birth in the male, degeneration of this zone begins as a line of demarcation between it and the reticular part of the fasciculate zone. At 35 days, the degeneration has materially spread inwards, and by the 37th day the interlocking zone has almost ceased to exist as an organized layer. The process of degeneration is clearly an extension of that described for 30 days and consists of a shrinking of the nuclei and the disorganization of the cytoplasm. In sections of the gland at 37 days, stained with Mallory's triple stain, this degenerated layer is bright blue. However, at this age, the sections still show persistent normal cells in this layer. One preparation of a gland 42 days after birth, stained with haematoxylin and eosin, gives a very clear picture of what is taking place (fig. 8, Pl. 17). The interlocking zone is represented by nuclei, about four deep radially, all interlaced with highly eosinophil cytoplasmic strands. All the nuclei are small and collapsed but still stain intensely with haematoxylin. It is clear that this zone is completely degenerating and giving place to a connective tissue layer.

Outside this degenerating layer there is undoubtedly a layer to be distinguished as the zona reticularis. The cells of this layer are shorter than the fasciculate cells, and although having approximately the same cytoplasmic contents they are undoubtedly a little denser and take the eosin stain more deeply. There is considerable individual variation in the arrangement of this layer. Thus at 30 days after birth the definite beginning of a reticulate layer was present evidently differentiating from the innermost region of the fasciculate zone (fig. 7, Pl. 17). In some adrenals of 37 days this has developed in size, shape, and irregularity of cells until a typical reticular structure, with abundant blood-cells, exists outside the connective tissue layer. On the other hand, that part of the section of a 42-days gland chosen to illustrate the degeneration of the interlocking zone (fig. 8, Pl. 17) shows little evidence of a reticular layer. The layer is visible, however, in other parts of the same section.

The fasciculate zone consists of radial columns of cells between which are blood-vessels. The cells have all approximately the same width but vary in length. This variation in length is regular, the cells adjacent to the zona glomerulosa being the shortest, the length increasing in the cells nearer to the centre of the gland. This heightens the impression that division of the cells takes place in the glomerular zone, where the nuclei are almost touching each other, and that the products of division pass inwards, forming the columns of the fasciculate zone.

The zona glomerulosa has now the characteristic arched structure, that is, so far as it is ever attained in the mouse.

The structure of the cortical nuclei remains as in earlier stages.

The medulla itself remains in exactly the condition described in the previous stage. The cell size has remained constant since about 30 days after birth, and the cytoplasm is still distinctly basiphil and diffuse in the cell. With regard to the portions of the interlocking zone isolated within the medulla (cf. p. 357) there is considerable individual variation, some show absolutely none of this tissue while others show a considerable quantity even at 56 days.

#### 42-56 Days in the Male.

Although there is considerable individual variation, the degeneration of the interlocking zone is completed in all males during this period. The 4-8 layers of crushed nuclei with their attendant cytoplasm concentrate into a connective tissue sheath which then completely separates the medulla from the remaining outer part of the cortex. The region of the cortex immediately external to the connective tissue sheath is now a distinct reticular zone (see fig. 9, Pl. 17).

This appears to be the final developmental stage and to be the mature condition in the male.

The gland now consists of a well-developed medulla with cells arranged in groups of 6-12, in section, bound together by a connective tissue layer. The nuclei are of the two kinds already described—of essentially the same structure but one type distinctly more granular and slightly smaller than the other. In the spaces amongst the cell groups are large blood sinuses,

and in many glands are still to be seen remains of the stranded cortical tissue. My material is not sufficiently extensive to enable me to judge whether or not this tissue is eventually entirely lost.

Separating the medulla from the cortex is a well-marked connective tissue capsule, and immediately external to this is a reticular zone with blood spaces between the cells. Continuous with the cells of the reticular zone are the rectangular cells which constitute the fasciculate columns, and these radial columns in their turn are continuous with the 'arched' groups of cells which form the zona glomerulosa. Surrounding the whole structure there is a fibrous capsule.

#### Note on the Development of the Adrenal Gland of the Female after 35 Days.

This investigation has not dealt in detail with the condition of the adrenal gland in the female mouse after the age of 35 days. The attainment of maximum development of the interlocking zone, its slow involution in virgin life and its accelerated degeneration in the early stages of pregnancy are described in the published observations of Masui and Tamura (1926), Tamura (1926), Miller (1927), Deansley (1928), and Whitehead (1933, *a*).

Such observations as I have made are in harmony with the findings of these authors. However, certain points of considerable interest appear to be insufficiently illustrated. It is quite clear that the region which degenerates is homologous in the two sexes, although fatty degeneration, as found in some females (fig. 10, Pl. 18), is never found in the male. Also, as Whitehead (1933, *a*) points out, because the interlocking zone reaches greater dimensions in the female than it ever does in the male, the connective tissue band which remains after its degeneration is correspondingly more extensive in the female (cf. fig. 11, Pl. 18, with fig. 9, Pl. IV).

#### IV. DISCUSSION.

As was pointed out at the beginning of the present paper, many different workers have recorded degeneration of an inner region of the adrenal cortex of the mouse associated with

various phenomena. In the female, fatty or non-fatty degeneration of the inner cortical region is said to take place during pregnancy or, in the absence of pregnancy, during advanced mature life. In the male, degeneration of an inner region is recorded as taking place just before maturity. It is not clear whether degeneration is of the same type under these different circumstances.

There is also some confusion in regard to the identity of the zone which degenerates. Some speak of the whole degenerating region as the reticular zone (Masui and Tamura), while others speak of it as the X-zone (Miller). On the other hand, the degeneration is sometimes said to involve only the inner part of the X-zone, the outer part giving rise to a reticular zone which does not degenerate (Deansley). Miller also recognized this zona reticularis as being present after the degeneration of the X-zone, but considered that it represented the inner ends of the fasciculate columns.

In the cat there is no degeneration of any part of the cortex in the adult, but in young animals an inner region of the cortex does degenerate and finally disappears at about 2 or 3 months (p. 330). This inner region interlocks with the medulla and is part of the original cortical anlage. At a very early stage the cortical anlage consists of a clearly defined peripheral zone and an apparently undifferentiated inner mass. It is this inner mass which later forms the zone immediately around the medulla and interlocking with it, and this zone gradually becomes less and less during early post natal life and disappears completely at about 3 months. A reticular zone, differentiating from the inner part of the already well-defined fasciculate zone, is evident just before the inner zone finally disappears.

From its history this inner zone of the cat adrenal is almost certainly homologous with the boundary zone of the human gland.

In the light of these facts the possibility suggests itself that the inner zone of the mouse adrenal, which degenerates prior to maturity in the male and evidently also disappears, though much later, in the female, might possibly be the homologue of the boundary zone of man and of the cat, and as such might be an entirely separate structure from the reticular zone.



The facts brought to light in the present investigation appear to justify this speculation.

The interlocking eosinophil zone of the developing mouse adrenal is undoubtedly the remains of the original cortical anlage, as is the boundary zone in man and in the cat. In all these cases, also, the tissue is highly eosinophil, has highly granular cytoplasm and interlocks throughout its existence with the medulla. In man and the cat it is described as syncytial in nature and in the mouse the cell boundaries are inconstant in appearance. There can be little doubt that the three structures are homologous.

Also, in the development of the mouse the reticular zone has been definitely found to develop from the inner cells of the already partly differentiated fasciculate zone, not from the interlocking zone. The separate identity of the reticular and interlocking zones is beyond question. [Nevertheless, apart from a detailed knowledge of the different stages of development it would be very easy to confuse the two tissues, for both are more eosinophil than the rest of the cortex.]

Further, the interlocking or boundary zone in the mouse undergoes clear degeneration just before maturity in the male. The degeneration is not of the fatty type, but consists of a gradual shrinking and crushing of the nuclei accompanied by general collapse of the cells. This collapsed tissue forms a connective tissue sheath separating the medulla from the reticular zone of the cortex.

#### V. SUMMARY.

1. The cortical anlage is constituted from the mesenchyme at about the 12th day of embryonic life.
2. The medullary anlage is constituted at about the 18th day of embryonic life from the anlage of the sympathetic nervous system and lies closely adpressed to the cortical anlage.
3. At about the 14th day sympatho-chromaffin elements migrate from the closely adpressed sympathetic nervous mass into the cortical anlage and form irregularly arranged 'nests' in its substance.
4. Between 16 and 18 days of foetal life part of the cortical

substance has differentiated by becoming less eosinophil and is destined to form the adult permanent cortex.

5. By the day of birth the sympatho-chromaffin elements are concentrated at the centre of the gland and are beginning to metamorphose into medullary cells.

The undifferentiated eosinophil cortical tissue now forms a layer immediately outside the medulla and interlocking with the medullary tissue.

The less eosinophil tissue now shows the first distinct signs of the arrangement characteristic of the adult glomerular and fasciculate zones.

6. After birth, in both sexes, the interlocking zone increases, attaining its maximum at about 21 days in the male but continuing to increase in the female, so that at about 25 days there is a distinct sex difference.

The three zones of the adult cortex are distinguishable in both sexes by 25 days—the glomerulosa, zona fasciculata, and zona reticularis.

The reticular zone is distinctly a product of the zona fasciculata and not of the interlocking eosinophil zone.

7. At 30 days in the male degeneration of the interlocking zone has begun—in the female the zone is still increasing. The male reticular zone is now very clearly developed but that of the female has remained at the 25-days condition.

8. At 35 days in the male degeneration of the interlocking zone has become well established. In the female this zone attains its maximum development at about this period.

9. By 56 days in the male the degeneration of the interlocking zone is complete, leaving a connective tissue capsule between the medulla and the well-marked zona reticularis of the permanent cortex. (The female is not discussed beyond 35 days.)

10. The interlocking zone of the adrenal of the mouse (X-zone of Miller) is shown to be probably homologous with the boundary zone of man and of the cat.

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## DESCRIPTION OF PLATES 14-18.

Unless otherwise mentioned all figures are magnified according to scale on Plate 17.

## PLATE 14.

Fig. 1.—Drawing of a transverse section of the adrenal gland and applied splanchnic plexus of a 14-days embryo. (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

## PLATE 15.

Fig. 2.—Drawing of a transverse section of the adrenal gland and applied splanchnic plexus of a 15-days embryo, showing a definite nerve connexion. (Ciaccio's fluid: Ehrlich's haematoxylin and eosin.)

Fig. 3.—Drawing of part of a transverse section of the adrenal gland of a 15-days embryo to show mitotic figures and the distinctive features of the sympatho-chromaffin and cortical nuclei. Magnified according to scale on Pl. 17  $\times 2$ . (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

## PLATE 16.

Fig. 4.—Drawing of part of a transverse section of the adrenal gland of a late 17-days embryo to illustrate the differentiation of the cortical substance. (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

Fig. 5.—Drawing of part of a transverse section of an adrenal gland on the day of birth.  $\times$  half scale on Plate 17. (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

Fig. 6.—Drawing of part of a transverse section of an adrenal gland 18 days after birth. (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

## PLATE 17.

Fig. 7.—Drawing of part of a transverse section of a male adrenal gland 30 days after birth. (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

Fig. 8.—Drawing of part of a transverse section of a male adrenal gland 42 days after birth. (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

Fig. 9.—Drawing of part of a transverse section of a male adrenal gland 50 days after birth. (Bouin's fluid: Mallory's triple stain.)

## PLATE 18.

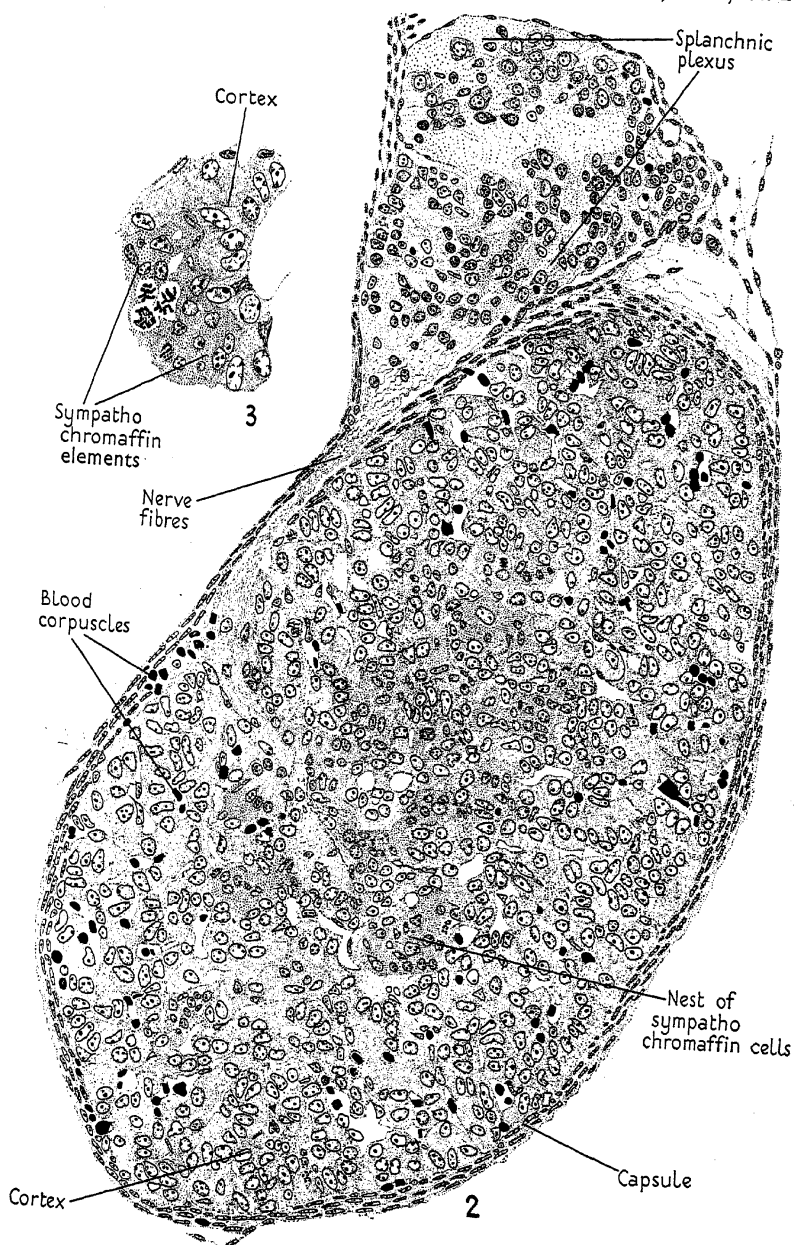
Fig. 10.—Drawing of a transverse section of an adult female adrenal gland showing extensive vacuolization of the interlocking zone. (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

Fig. 11.—Drawing of a transverse section of an adult female adrenal gland showing the connective tissue band remaining after the collapse of the interlocking zone (14th day of pregnancy). (Bouin's fluid: Ehrlich's haematoxylin and eosin.)

Fig. 12.—Semi-diagrammatic figure to show the relationship of the organs in a transverse section of a 14-days embryo.  $\times$  according to  $\frac{1}{3}$  scale on Plate 17. (Bouin's fluid and Ehrlich's haematoxylin.)

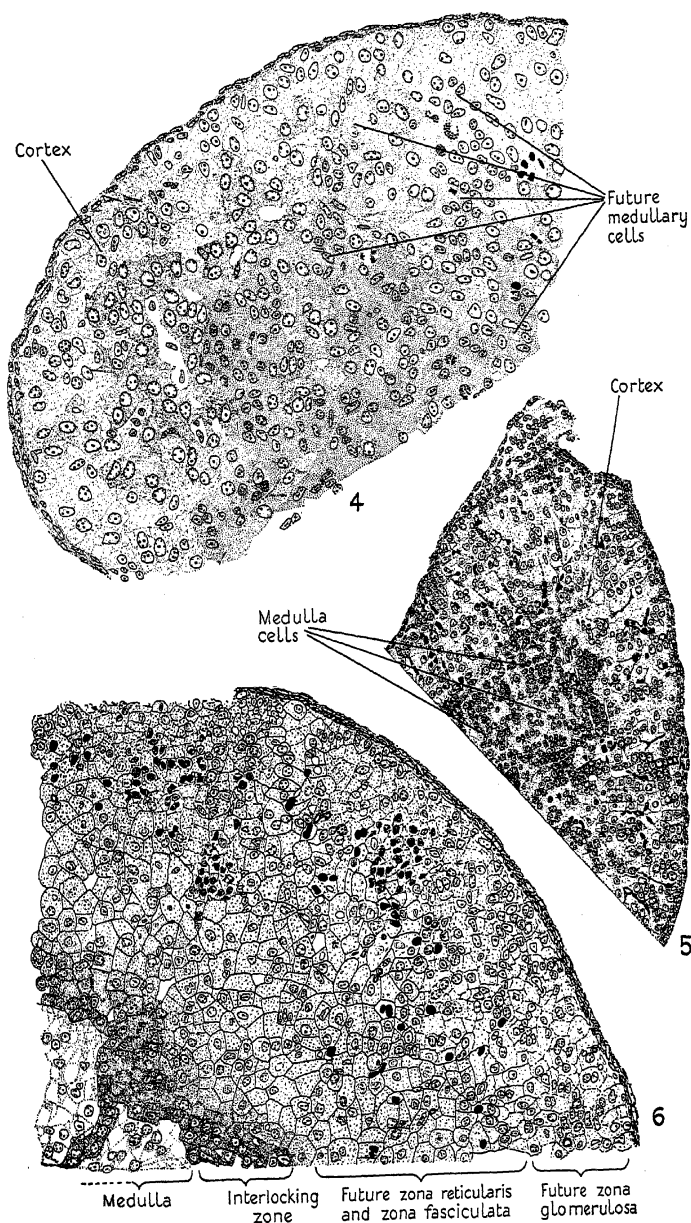




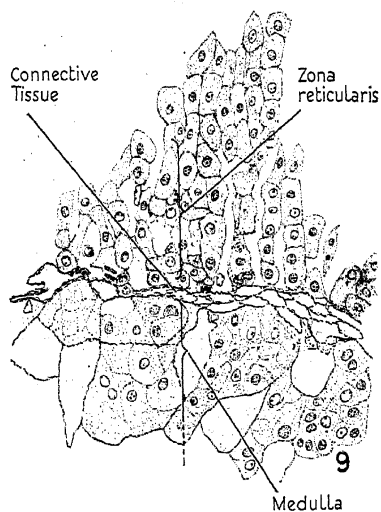
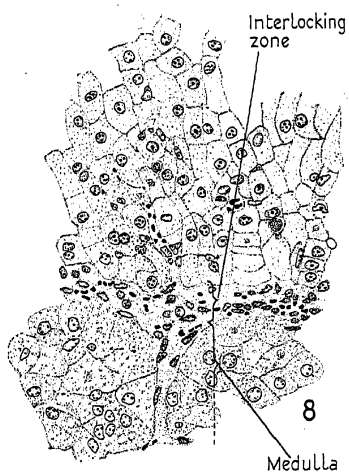
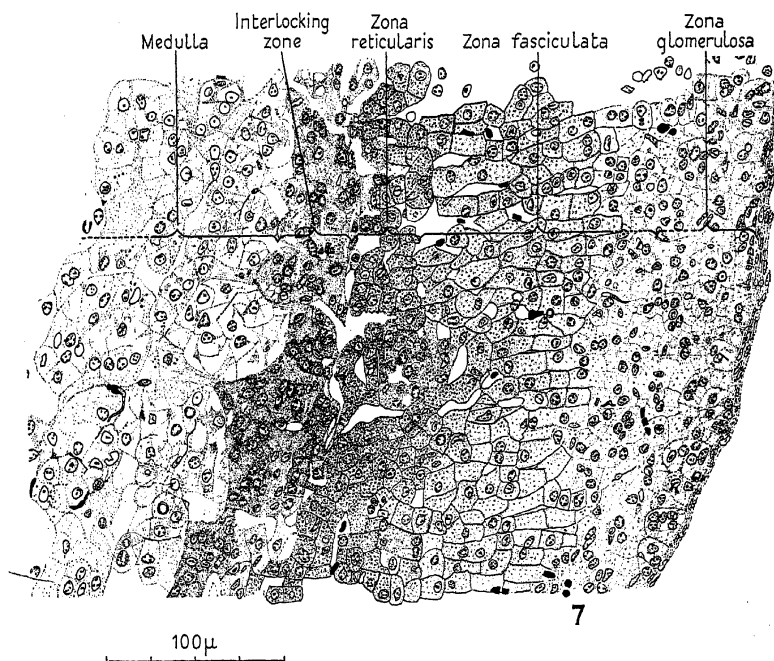




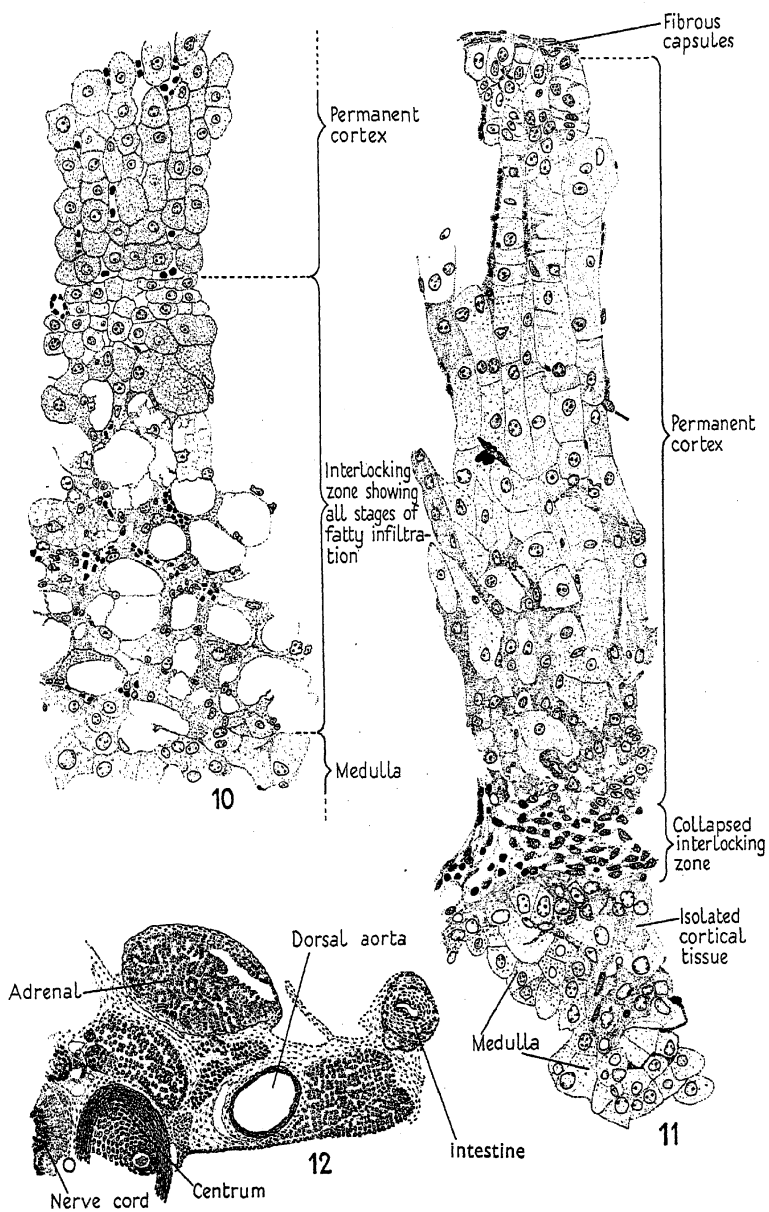












*H. Waring, del.*



# The Giant Nerve Fibres and Epistellar Body of Cephalopods.

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With 20 Text-figures.

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## INTRODUCTION.

At the hind end of the stellate ganglion of *Eledone moschata* there is a small yellow spot, about the size of a pin's head, which can be seen on examination of serial sections to consist of a closed vesicle. On account of its position it has been named the epistellar body (Young, 1929). It has been found in all the Octopods examined, though it is not always coloured yellow. Search was made for a similar organ in the same position in Decapod Cephalopods but no trace of it could be found. However, during the search certain extremely large nerve fibres were noticed in the stellate ganglion of *Loligo*, and study of these showed them to originate not from giant nerve cells, but by the fusion of the axons of a very large number of small cells which are congregated together in a separate lobe situated at the hind end of the ganglion. This lobe lies in a position which corresponds exactly to that of the epistellar body present in Octopods, and these latter have no giant fibres. The conclusion which has been drawn as a result of study of the two systems is that the epistellar body of Octopods is derived from the cells of the giant fibres of Decapods; and, since the former is probably secretory, we have



here a case of the formation of gland from nerve cells parallel to that of the adrenal medulla of Vertebrates. The many curious features of this peculiar transformation are described in this paper, beginning with a preliminary account of the giant fibres themselves, which present several points of very great interest.

The material described has been collected over several years, during visits to the Stazione Zoologica di Napoli and the Marine Biological Station at Plymouth.<sup>1</sup> Nearly all the observations have been made on sections fixed in various ways, which are detailed in the text. The silver methods used for the study of the details of the nervous connexions have been described elsewhere (Sereni and Young, 1932). The formol-Cajal method gives particularly good stains of the finest branches of the giant fibres.

#### GIANT NERVE FIBRES IN DECAPODS.

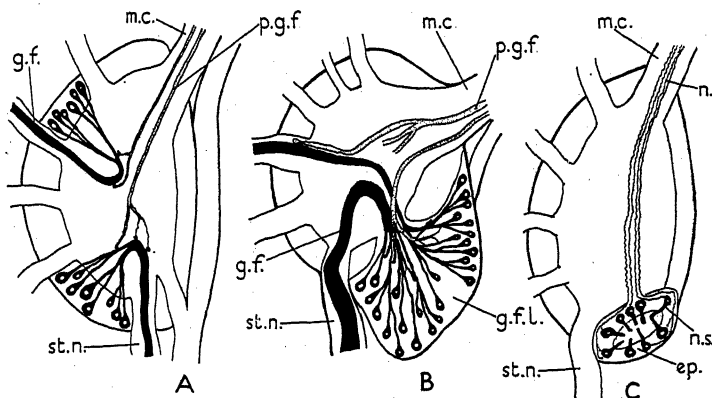
Giant nerve fibres have been found in several groups of cold-blooded animals, their function being apparently the conduction of impulses for the performance of some rapid action of advance or retreat involving the synchronous contraction of large numbers of muscles. Thus it is by means of their giant fibres that Earthworms are enabled to retract rapidly when touched either at the head or tail end (Stough, 1926).

To the best of my knowledge the only mention of giant fibres in Cephalopods, or indeed in any Mollusc, is the very brief account of Williams (1909), who noticed the giant fibres in the central nervous system of *Loligo pealii*. He believed that these ran straight into the pallial nerve and thence through the stellate ganglion into the stellar nerves. In *Loligo forbesi* this is certainly not correct. The giant fibre system begins with a pair of giant cells lying at the hind end of the pedal ganglion. The processes of these cells pass backwards into the pallio-visceral ganglion, in which the two axons actually fuse across the middle line, instead of merely crossing as described by Williams. They then separate again and each breaks up into several branches which end in synaptic junctions with other giant fibres arising from cells in the palliovisceral ganglion and

<sup>1</sup> I am grateful to Dr. E. J. Allen and to Dr. R. Dohrn for their help, also to Professor E. S. Goodrich for criticism of the MS.

passing out in the posterior infundibular, visceral, and pallial nerves.

The details of the anatomy of this giant fibre system will be published later, here it is sufficient to say that the fibres running backwards in the pallial nerve do not, as supposed by Williams,



TEXT-FIG. 1.

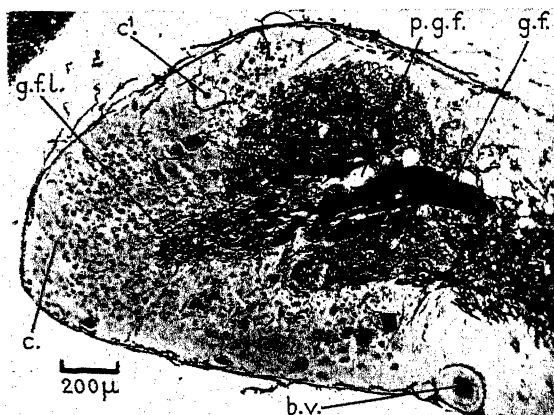
Diagrams of stellate ganglia of Cephalopods. In *Sepia* (A) the giant fibres arise from cells scattered throughout the ganglion, and in *Loligo* (B) from cells collected into a giant fibre-lobe. In *Octopods* (C) there are no giant fibres, but in the position of the giant fibre-lobe are the cells whose axons end blindly in the epistellar body.

#### LETTERING FOR TEXT-FIGURES 1-20.

*am.*, amoebocyte; *b.*, terminal bouton; *b.v.*, blood-vessel; *c.*, cell of giant fibre-lobe; *c'*, ordinary cell of stellate ganglion; *cav.*, cavity of epistellar body; *c.ep.*, epithelial cells of epistellar body; *ep.*, epistellar body; *f.*, connective tissue fibrils; *g.*, osmiophil granules; *g.f.*, 'post-ganglionic' giant fibres; *g.f.l.*, giant fibre lobe; *h.*, homogeneous substance at centre of epistellar body; *l.*, isolated masses at centre of epistellar body; *m.c.*, mantle connective (pallial nerve); *m.ret.*, musculus retractor capitis; *m.m.*, mantle muscles; *n.*, nerve to epistellar body; *n.am.*, nucleus of amoebocyte; *n.conn.*, nucleus of connective tissue; *n.s.*, neurosecretory cell; *n.n.s.*, nucleus of neurosecretory cell; *p.n.s.*, process of neurosecretory cell; *p.g.f.*, 'pre-ganglionic' giant fibre; *st.g.*, stellate ganglion; *st.n.*, stellar nerve.

run right through the stellate ganglion, but end there in synaptic junction with another set of giant fibres, which we may call 'post-ganglionics', running out in the stellar nerves to innervate

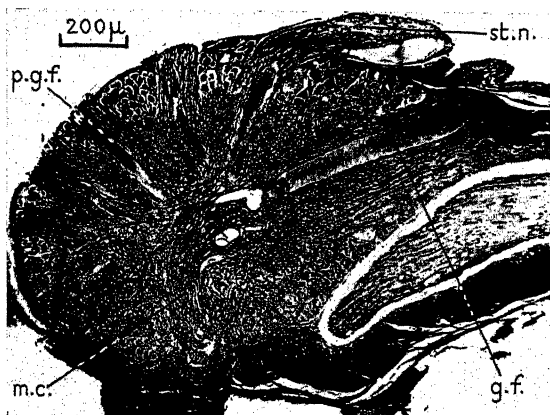
the mantle muscles (Text-fig. 1). The giant synapses in the stellate ganglion, by which the pre- and post-ganglionic fibres communicate, are of the greatest interest and will be described fully in a later paper; here we are concerned only with the post-ganglionic fibres themselves. These are of extremely large size, up to  $600\mu$  in diameter in *Loligo forbesi* and  $150\mu$  in *Sepia officinalis*. In *Loligo forbesi* there is one



TEXT-FIG. 2.

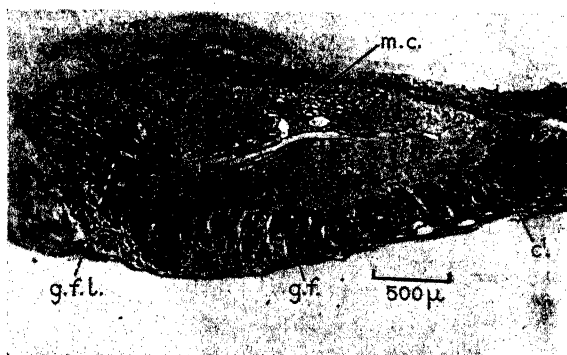
*Loligo forbesi*. Sagittal section of stellate ganglion showing giant fibres. Formol-Cajal.

of them in each of the stellar nerves, and if any one fibre is traced into the ganglion it is found to branch many times until the finest branches can be followed back to their origin as the axons of single cells situated in a separate lobe at the hind end of the stellate ganglion (Text-figs. 1, 2, 3, and 4). These giant fibres are therefore syncytia, each formed by the fusion of the processes of very many small nerve cells. It is not easy to say exactly how many cells go to the making up of each giant fibre. The number of post-ganglionic giant fibres varies in different individuals of *Loligo forbesi* between nine and fifteen, and estimates of the number of cells in the giant fibre lobe, obtained by counting the cells in a small area, vary between 5,000 and 15,000. It would appear, therefore, that the processes



TEXT-FIG. 3.

*Loligo forbesi*. Horizontal section through stellate ganglion, showing the giant fibres. Formol-Cajal.



TEXT-FIG. 4.

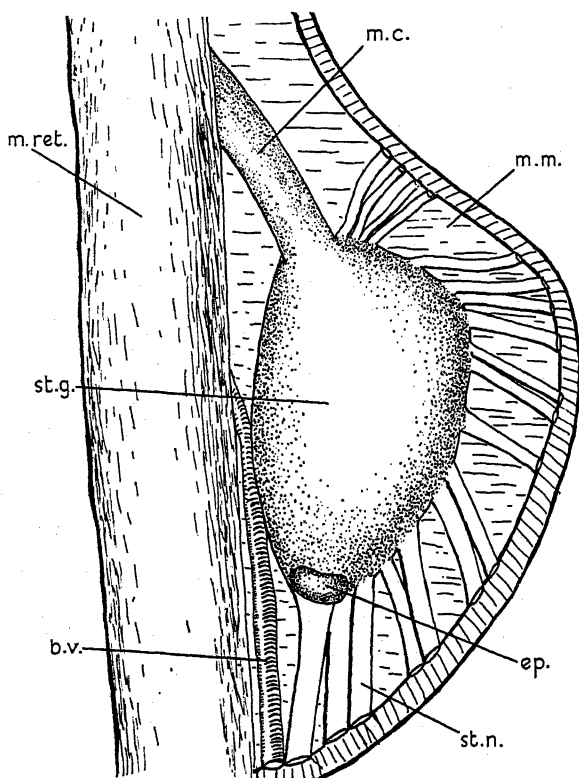
*Loligo forbesi*. Sagittal section of stellate ganglion showing giant fibres and the lobe from which they arise. Carnoy, toluidin blue.

of from 300 to 1,500 cells fuse to form each giant fibre. Since the latter are of different sizes it is probable that some arise from more, others from fewer cells.

This arrangement, though certainly very peculiar, is not unique in the animal kingdom. Thus several cell bodies contri-

bute to each of the segmental giant fibres of earthworms Stough (1926), and Speidel (1933) has actually seen the anastomosis of living axons in the tails of frog tadpoles.

In *Sepia officinalis* the giant fibres of the stellate gang-



TEXT-FIG. 5.

*Eledone moschata*. Right stellate ganglion seen from below, showing the position of the epistellar body.

lion arise as do those of *Loligo*, by fusion of the processes of a number of nerve cells which, however, are not all collected together into a single giant fibre-lobe, but are scattered throughout the ganglion (see Text-fig. 1). This is possibly the more primitive arrangement, from which the giant fibre-lobe has

arisen by collection together of all the cells which give rise to the giant fibres.

### THE EPISTELLAR BODY OF OCTOPODS.

#### (i) General.

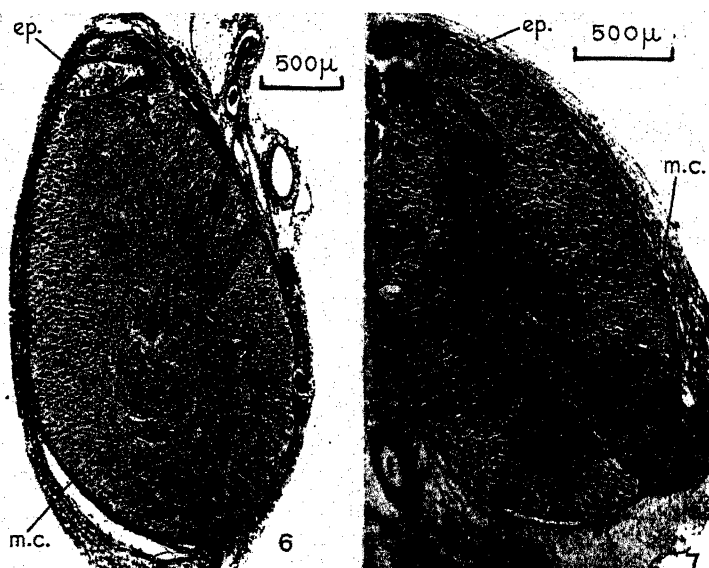
The epistellar body is a closed vesicle which has been found in all the Octopods examined, namely:

- Eledone moschata* (Leach).
- Eledone cirrosa* (d'Orbigny).
- Octopus vulgaris* (Lam.).
- Octopus macropus* (Risso).
- Octopus defilipi* (Vérany).
- Octopus salutii* (Vérany).
- Tremoctopus violaceus* (delle Chiaje).
- Argonauta argo* (L.).
- Ocythoe tuberculata* (Rafinesque).

In all these species the body lies at the hind end of the stellate ganglion, but it is pigmented only in *Eledone moschata*, *Eledone cirrosa*, *Octopus salutii*, and *Octopus macropus*, being difficult to make out with the naked eye in the other species. The organ is built on fundamentally the same plan in all these forms, but there are considerable and characteristic differences between the species. In the case of *Eledone moschata* and *Octopus vulgaris* epistellar bodies from numerous individuals were studied and some evidence of a cycle of activity was discovered.

A description will first be given of the conditions in *Eledone moschata*. It was in this animal that the body was first noticed, on account of the fact that it contains yellow pigment which was observed by Bauer (1908), who refers to a 'pigment-fleck' at the hind end of the stellate ganglion of *Eledone*. As can be seen from Text-figs. 5, 6, and 7 the body lies on the outer side of the ganglion, close to the origin of the hindermost and largest stellar nerve, and not far from the large artery which runs along the side of the ganglion. The body is of an irregular oval shape, with its long axis transverse to that of the body.

Serial sections show that it is shallow in proportion to its exposed surface area, the dimensions in one specimen being  $500\mu$  across and  $75\mu$  deep. The form is irregular and shows considerable variation. Usually the organ contains a single main cavity having several diverticula (Text-fig. 8), but occasionally



TEXT-FIGS. 6-7.

Fig. 6.—*Eleidone moschata*. Sagittal section of stellate ganglion, showing epistellar body with two cavities. Bouin, azan. 9.2.29.

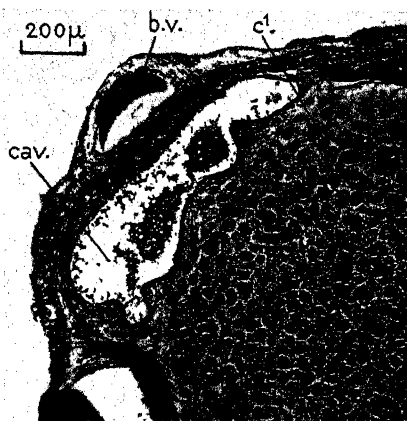
Fig. 7.—*Octopus vulgaris*. Sagittal section of the stellate ganglion, showing the position of the epistellar body. Bouin, Ehrlich's haematoxylin. 7.1.29.

there are two completely separate cavities (Text-fig. 6). The organ usually lies within the thick connective tissue sheath which surrounds the ganglion, but is isolated from the latter by a thin layer of connective tissue, broken at one place to allow of the entrance of a nerve. Occasionally the body is quite separate from the ganglion and enclosed in its own connective tissue sheath.

(ii) The Neurosecretory Cells.

The walls of the epistellar body may be said to consist of three layers:

- (1) The connective tissue referred to above.
- (2) An irregular layer of medium-sized cells, having long processes which extend into the cavity of the organ.
- (3) An inner layer lining the cavity, and consisting of small epithelial cells between which pass the processes of the cells of the middle layer.



TEXT-FIG. 8.

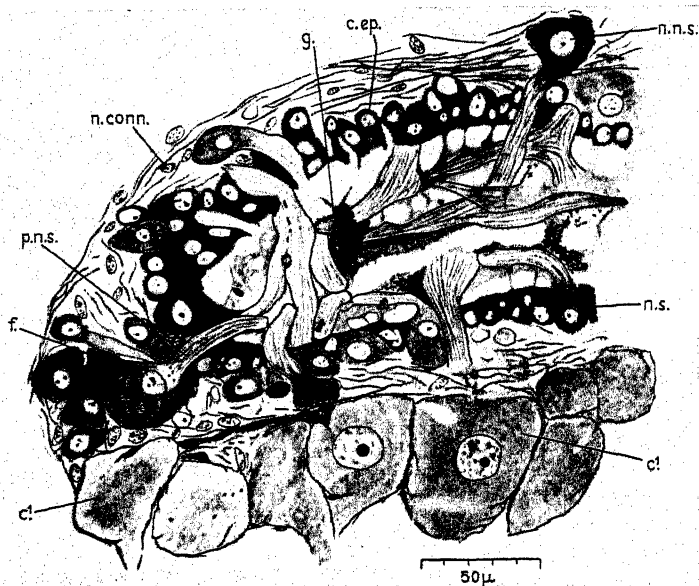
*Eledone moschata*. Sagittal section of the epistellar body, showing the irregular shape of the central cavity. Zenker, carmalum, picronigrosin.

It is the second of these types of cells which constitute the most peculiar feature of the body (Text-figs. 9, 10, and 11). They have rather large nuclei with several karyosomes, and a distinct layer of cytoplasm, the latter containing a few rather large granules which stain well with iron haematoxylin after fixation in Flemming's fluid. Round the outside of these cells are numbers of fibrils, apparently of connective tissue, which stain very readily with basic stains (Text-fig. 9). The cells are elongated at right angles to the wall of the organ, having a long process which



enters the cavity. Sometimes they also have one or two shorter processes directed away from the centre of the body.

The processes of these cells which enter the cavity very much resemble the axons of nerve cells, having, in fixed preparations, a faintly striated appearance (Text-fig. 9). These processes



TEXT-FIG. 9.

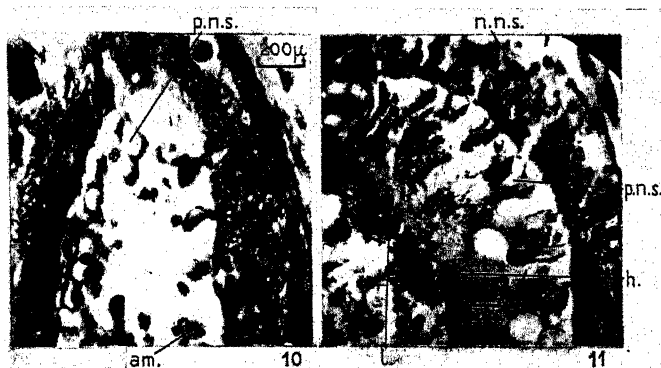
*Ocythoe tuberculata*. Sagittal section of epistellar body.  
Camera lucida drawing with W.W.1/12" oil imm. Flemming,  
iron haematoxylin. 20.3.30.

usually end blindly, embedded in a homogeneous substance which fills the cavity.

In preparations stained with Cajal's method it can be seen that small darkly staining knobs, closely resembling the boutons terminaux of the neuropil (Sereni and Young, 1932), lie close to the outer ends of these bipolar cells (Text-fig. 12). These boutons are the terminals of a small nerve which enters the epistellar body from the neuropil of the stellate

ganglion (Text-fig. 13). In serial sections this nerve can be traced through the ganglion to the pallial nerve (mantle connective): and in experiments in which the latter had been cut for short periods before death, it was found that the fibres which run to the epistellar body were degenerating.

It will be seen, therefore, that the cells whose processes enter



TEXT-FIGS. 10-11.

Fig. 10.—*Octopus vulgaris*. Epistellar body, showing inner ends of the neurosecretory cells. Flemming, iron haematoxylin. 15.5.29.

Fig. 11.—*Eledone moschata*. Epistellar body showing neurosecretory cells and contents of the cavity. Bouin, azan. 9.2.29.

the cavity of the epistellar body have many points of similarity with the neurons of the stellate ganglion. Besides having long processes which immediately recall axons, they also resemble the nerve cells in the structure of their nucleus and cytoplasm and in being innervated from the mantle connective. That they are indeed modified neurons is confirmed by the fact that in some cases there can be seen side by side ordinary neurons of the ganglion and the cells here described, so that it is sometimes difficult to decide which is which. This transition is only rarely visible in *Eledone* on account of the thick sheath of connective tissue, but it appears very clearly in *Tremoctopus* (Text-fig. 14). Since these cells appear to be of nervous origin



TEXT-FIGS. 12-13.

Fig. 12.—*Eledone moschata*. Part of epistellar body, showing nerve ending on outer prolongation of neurosecretory cell. Formol-Cajal. 9.2.29.

Fig. 13.—*Eledone moschata*. Sagittal section of epistellar body, showing its nerve. Bouin, azan. 4.8.31.



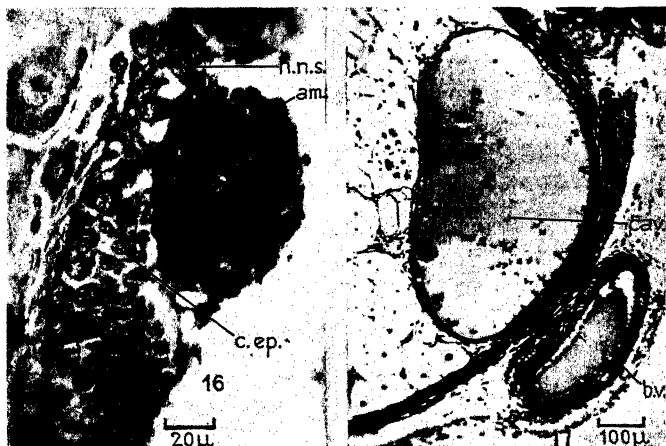
TEXT-FIGS. 14-15.

Fig. 14.—*Tremoctopus violaceus*. Epistellar body, showing similarity between neurosecretory cells and neurons. Bouin, iron haematoxylin.

Fig. 15.—*Argonauta argo*. Sagittal section through epistellar body. Bouin, Ehrlich's haematoxylin, eosin. 11.7.30.

and, as will be shown below, they probably have a secretory function, it is proposed to call them neurosecretory cells.

The third set of cells mentioned on p. 375 as forming a lining to the cavity resemble the neurosecretory cells in the structure



TEXT-FIGS. 16-17.

Fig. 16.—*Octopus vulgaris*. Part of epistellar body, showing fused inner ends of the neurosecretory cells. Flemming, iron haematoxylin. 22.6.29.

Fig. 17.—*Octopus macropus*. Swollen epistellar body. Bouin, azan. 19.7.29.

of their nuclei, and it is possible that they, too, represent neurons which have become still further modified.

It remains to describe the conditions in the cavity at the centre of the epistellar body. It is here that the greatest differences are seen between individuals of a single species, differences which are perhaps correlated with some cycle of activity. In some animals the wall of the body appears stretched and the cavity large and mainly filled by an optically homogeneous substance (Text-fig. 17). This latter stains readily with nigrosin or anilin blue, but not with basic dyes, or with any of the usual acidic stains such as eosin, orange G, or acid fuchsin. Embedded in the outer edges of this substance lie the inner ends of the

neurosecretory cells (Text-figs. 9, 10, and 11). Sometimes there are masses of osmiophil granules collected round the ends of these cells, possibly representing a secretory product. The only other constituents of the cavity in this state are a few amoebocytes which lie near the centre of the homogeneous substance and usually contain large granules which stain readily with osmium tetroxide.

Frequently the contents of the cavity are more complex. The homogeneous substance is broken up and interspersed with large, irregular lumps containing fibrillae which stain deeply with basic dyes, and resemble those seen round the outer edges of the neurosecretory cells (see p. 375). So far as can be ascertained these masses consist of the shrunken inner ends of the neurosecretory cells which have become nipped off from the cell body. Only very rarely is the nucleus of one of these cells seen inside the cavity. The shrunken remnants often occur in large numbers scattered throughout the cavity, and each such mass may be surrounded by one or more amoebocytes, which appear to be devouring it by phagocytosis.

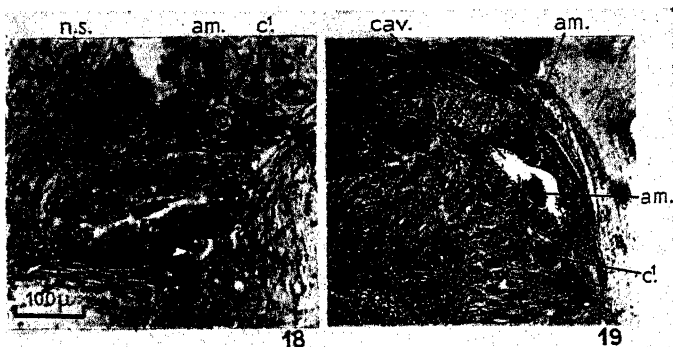
All gradations are seen between this and the third stage, in which the walls are relaxed and the cavity very small. The number of amoebocytes is very much increased, so that they almost fill the cavity. The homogeneous substance has almost disappeared, and the only other contents besides the amoebocytes are the processes of the neurosecretory cells, apparently fusing at the periphery of the mass of amoebocytes to form a syncytium (Text-fig. 16). The extreme of this condition was observed only in *Octopus vulgaris* taken in June, July, and August (Text-fig. 18). It is impossible to be certain whether it occurs also in *Eledone*, since this species is not to be found during the summer months in the bay of Naples as it migrates into deep water to breed. In two of the *Octopus* in question not only was there a mass of amoebocytes filling the cavity, but they could also be seen to be bursting through its wall (Text-fig. 19); whether they were passing inwards or outwards could not be determined.

There appears, therefore, to be some seasonal change in the contents of the body, especially in the sense that there are more

amoebocytes during the summer months. But at no time is the cavity quite devoid of amoebocytes, and it seems more than likely that the differences observed are due, not to a cycle of activity, but simply to the much more rapid course of all processes during the summer months.

(iii) Function of the Epistellar body.

The only evidence at present available as to the function of the epistellar body was collected in 1929 before morphological



TEXT-FIGS. 18-19.

Fig. 18.—*Octopus vulgaris*. Epistellar body with very small cavity, filled with amoebocytes. Flemming, safranin. 19.6.29.

Fig. 19.—*Octopus vulgaris*. Epistellar body, showing amoebocytes passing through the wall. Flemming, safranin. 22.6.29.

studies had provided the suggestion that it consists of modified nerve cells. The late Professor Sereni was kind enough to assist me in removing both epistellar bodies from nineteen *Eledone moschata*. The operation itself, performed under urethane anaesthesia, is a very slight matter for the animal,<sup>1</sup> and yet after it abnormalities were seen, which may best be described as a state of general muscular atonia. Instead of moving actively about the tanks the operated animals remained attached to the sides, with the tentacles hanging limply, giving a striking picture

<sup>1</sup> Other minor and major operations, such as section of the stellar nerves and gonadectomy, served as controls in this respect.

of depression. Since the chromatophores of Cephalopods are kept expanded by muscles they provide an excellent index of the muscular tone. The animals from which the epistellar bodies had been removed became abnormally pale in colour, indicating a lack of tone in the muscles of the chromatophores.

This state of affairs lasted for about a week and then gradually passed off, the animals returning to an apparently normal condition in which they remained for as long as observed (up to 186 days). Histological examination, however, showed that no regeneration of the epistellar body had occurred.

From these results it is suggested as a working hypothesis that the epistellar body produces a secretion which assists in the maintenance of the muscular tone of the animal. It is natural to look for this product among the varied contents of the cavity. Perhaps the secretion is produced at the inner ends of the neurosecretory cells and diffuses away thence to the blood-stream. The granules surrounding the ends of the 'axons' of these cells, or the homogeneous substance which fills the cavity, suggest themselves as being perhaps the actual secretory product, but further evidence is required on this point.

The role played by the amoebocytes in the epistellar body is uncertain. They may assist in the transport of active secretion to the blood-stream, or simply in the removal of exhausted portions of the cells.

#### DISCUSSION.

##### Origin of the Epistellar Body from Giant Fibres.

The evidence on which it is suggested that the epistellar body is derived from the cells of the giant fibres may now be summarized. The epistellar body lies in the same morphological position as the giant fibre lobe of *Loligo*. The Decapods have giant fibres but no epistellar, whereas the Octopods have epistellar but no giant fibres. The giant fibre lobe is already partly cut off from the rest of the ganglion and the neuropil at its centre consists of fused processes of the cells of its walls; so that seen in certain aspects it is not at all unlike the epistellar body, in which the centre may also be filled by the fused processes of cells (Text-fig. 20). If the giant fibre lobe became

completely cut off it would resemble the epistellar body in all essential points. That the conducting function of the giant fibres should be lost in Octopods is in accordance with the general reduction which has taken place in the respiratory and locomotor functions of the mantle, these activities being performed more and more by the arms (Robson, 1931).

There can therefore be little doubt that the cells of the epistellar body are homologous with those which in *Loligo*



TEXT-FIG. 20.

*Loligo forbesi*. Transverse section of hind end of stellate ganglion, to show similarity of giant fibre lobe to epistellar body. Carnoy, toluidin blue.

give rise to the giant fibres, and since the evidence given above seems to show that the epistellar produces a secretion, this means that neurons have been converted into secretory cells. However, it must be remembered that the evidence as to the function of the epistellar body is still very incomplete, and it is possible that it represents simply a rudimentary vestige of the giant fibre lobe.

Such a transformation of nerve into gland is by no means unlikely on general grounds. The cells of the adrenal medulla of Vertebrates are derived from nerve cells, and nests of accessory chromophil cells occur in sympathetic ganglia. Gaskell (1914) claimed that in Annelids certain large neurons of the ventral ganglia contain pressor substances, revealed by the chromophil reaction which they give with bichromates. Recently



Hanström (1931, 1934) has described cells in the eye-stalks of various Crustacea which, though secretory, are probably of nervous origin. There is considerable evidence that nerve endings, in smooth muscle, exert their effects by the liberation at their ends of some stimulating substance (see Parker, 1932). There is no positive evidence that this is the case in Cephalopods, but it is perhaps significant that a betain is found in considerable quantities in the mantle muscles (Henze, 1910), and that this substance has been shown to have effects on the smooth muscles of the chromatophores similar to those of choline and its esters (Sereni, 1928).<sup>1</sup> It is tentatively suggested that in Decapods the giant fibres may exert their effects on the muscles by the liberation of some substance at the periphery, whereas in Octopods the fibres no longer run to the muscles but end blindly in the cavity of the epistellar body, where they produce a stimulating substance which is carried away in the blood-stream to the muscles. The evolutionary change necessary would not be a very large one, since the enzymatic or other system necessary for the synthesis of the stimulating substance would presumably be present at the peripheral endings of the giant fibres and thus be able to continue its work even when the fibres came to end in a closed vesicle.

However, this suggestion is clearly speculative, and needs the support of further experimental evidence, which I hope to collect as soon as material is available. The evidence in the present paper shows that the epistellar body contains cells of nervous origin which probably function by the production of secretion, but have yet retained something of their characteristic form as elongated neurons. They may thus be regarded as having reached a stage intermediate between ordinary motor-nerve fibres which liberate an active substance at the periphery, and the secretory cells of the adrenal medulla which though of nervous origin no longer bear any resemblance to neurons.

#### SUMMARY.

1. In Decapod Cephalopods there is a system of giant fibres probably serving to produce the rapid contractions of the mantle

<sup>1</sup> Bacq ('Nature', 136, 1935) has recently shown that acetyl choline is present in large amounts in the C.N.S. of Octopus.

muscles and ink-sac by means of which the animal shoots backwards behind a cloud of ink.

2. The giant fibres in the stellar nerves arise in the stellate ganglion, not from single giant cells, but as syncytia, by the fusion of the processes of a large number of cells. In *Loligo forbesi* all the cells giving rise to the giant fibres of the stellate ganglion are connected together into a giant fibre lobe.

3. In Octopods there are no giant fibres, but in the position of the giant fibre lobe there is a small closed vesicle, pigmented yellow in some species, and named the epistellar body.

4. In the walls of this body there are curious cells, the neurosecretory cells, whose general structure resembles that of neurons, but whose inner processes (axons) end blindly, embedded in a homogeneous substance which fills the cavity.

5. The neurosecretory cells are innervated by a small nerve which reaches them from the mantle connective.

6. After removal of both epistellar bodies from *Eledone moschata* the animal shows general muscular weakness for some days.

7. It is suggested that the epistellar body has arisen from the giant fibre lobe, and that the neurosecretory cells produce at their inner ends a secretion which is poured into the bloodstream.

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# Ultra-Centrifuging the Spermatocytes of *Helix aspersa*.

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With Plate 19 and 6 Text-figures.

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## INTRODUCTION.

SINCE the development of electric centrifuges giving rotation speeds about 5,000 R.P.M., a considerable amount of useful experimental work has been done, especially in eggs in which the granules can easily be shifted into layers. These centrifuges are unable to move the bodies in the nucleus or cytoplasm of the smaller somatic cells, and so the method has been restricted. Centrifuges rotating at higher speeds usually give trouble owing to the bearings burning out, the best instruments of this kind being run in nitrogen gas.

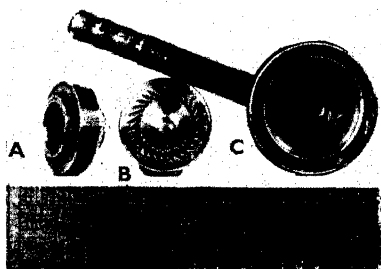
The development of the so-called 'ultra-centrifuge' by J. W. Beams, A. J. Weed, and E. G. Pickles opens a wide field for research in the study of somatic and germ cells. This centrifuge spins in a column of air, and so the trouble with bearings does not arise.

The evidence so far procured by means of the ultra-centrifuge in the investigations of Beams and King is of importance to those whose work in the cytoplasmic inclusions has been questioned, especially by Canti (6) and Walker and Allen (16), who hold that the Golgi apparatus is an artifact and 'due entirely to the action of the reagents used upon homogeneous cytoplasm, and that no structures of the kind exist in the living cell'.

## THE ULTRA-CENTRIFUGE.

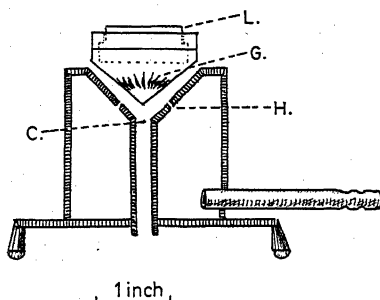
Accounts of the development of the ultra-centrifuge will be found in (3, 15). The type used by us is shown in Text-fig. 1, the size of the rotor (A, B) being quite small. In A it is seen

from the side, the screw top on the left; the inside of the rotor has been turned out hollow on a lathe, the objects to be centrifuged being placed inside. In B the grooves on the bottom of



TEXT-FIG. 1.

the rotor are shown, and in C the stator is shown. The pressure tubing from the source of air pressure is fixed in the tube on the left, and the compressed air emerges from eight or nine small



TEXT-FIG. 2.

staggered holes in the crater, hits the grooves of the stator, and makes the latter rotate in the air column so produced. In Text-fig. 2 a rough drawing indicates the structure of the crater (c), and attention may be drawn to the fact that the bottom of the cone or crater opens below, so that air passes up and around the spinning rotor. A smaller instrument of the same type as shown in the figures has been made to rotate as many as 2,000,000 revolutions a minute. The speeds ordinarily

used for centrifuging tissues have given 400,000 times gravity but owing to lack of a suitable compressed air plant in Dublin we have been obliged to use a motor-garage tyre-inflating plant, from which we got only about 50,000 times gravity. In the case of *Helix* cells this speed was quite enough for our purpose.

The stator is of brass, the rotors of steel, brass, or duralumin. The centrifuge is operated in a wooden box with thick walls to protect the operator in case the rotor should explode.

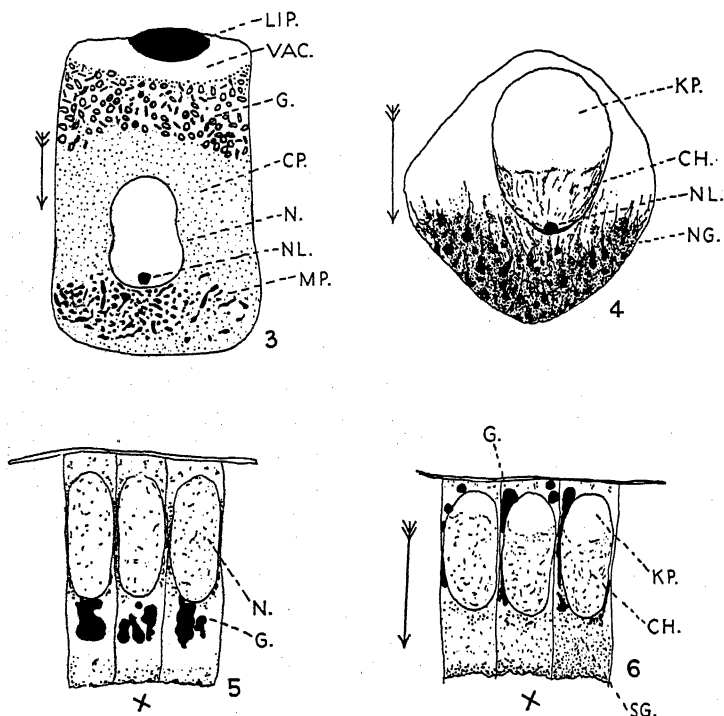
#### PREVIOUS WORK.

Browne (5) seems to have been the first to record the effect of centrifuging on the mitochondria of male germ cells. She found that in the centrifuged spermatocytes of *Notonecta*, the mitochondria are, in general, heavier than the surrounding cytoplasm and move to the centrifugal pole of the cells.

In 1924 F. W. R. Brambell carried out some work in this Department on the centrifuging of molluscan eggs. Work previous to this, especially that of Conklin, will be found mentioned in Brambell's paper. Brambell came to the conclusion that, in such eggs as *Helix*, *Limnaea*, and *Patella*, centrifuged for one half-hour (3,000–4,000 R.P.M., at an acceleration of 2,000 times gravity), three layers were produced, an upper layer of Golgi yolk (10 per cent.), a middle layer of clear cytoplasm, and a third or lower layer (50 per cent.) of swollen mitochondria, the unaltered Golgi elements remaining scattered through the cell. A full account of the literature on the effect of centrifuging upon the mitochondria and Golgi apparatus may be found in two recent papers (1, 2).

The introduction of the 'ultra-centrifuge' of J. W. Beams and his associates, as stated above, has enabled investigators to get speeds as much as 400,000 times gravity, and in Text-figs. 1–4 some of the results already reported by H. W. Beams and R. L. King (1, 2, 2a) are shown. In Text-fig. 3 is a bean root-tip cell showing an uppermost layer of lipoid (lip.), followed by a watery layer (vac.), next a layer of Bowen's bodies or Golgi apparatus (g), then a clear layer of protoplasm (cp.), and finally a lowermost layer containing mitochondria, pseudo-chondriome,

plastidome, and plastids (MP.). The nucleus (N.) is often drawn out to an ovoid, or bottle shape, the nucleolus (NL.) passing centrifugally. Undoubtedly the most remarkable fact brought out by this work is the position taken up by the osmiophilic



TEXT-FIGS. 3-6.

platelets of Bowen or Golgi bodies (G.). There is no seriation between the mitochondria at the bottom and the platelets above, as would be expected if the latter formed from the mitochondria as suggested by Guilliermond (11-13).

In Text-fig. 4 is shown a rat neurone ultra-centrifuged, and then stained so as to show the Nissl bodies (NG.) which pass centrifugally. The nucleus is divided into two layers, a clear (KP.) above and a chromatin mass (CH.) below, the lowermost position being taken up by a nucleolus (NL.).

In Text-figs. 5 and 6 are seen normal and ultra-centrifuged cells of the uterine glands of the guinea pig, X marking the lumen. The Golgi apparatus (g.) passes centripetally, squeezing past the nuclei in the narrow cells, and being pulled out to form streamers. Occasionally the end of the streamers break off, the resultant Golgi material forming spherical objects which do not mix with the cytoplasm. The nuclei also are made to form two layers, an upper (кр.) and a lower (сн.). The site of the Golgi apparatus (sg. in Text-fig. 6) is free of canals after the Golgi material has drifted upwards.

Recently E. Newton Harvey (15) has described an air-driven centrifuge by which the objects being centrifuged may be examined under the microscope while the centrifuging is taking place.

#### MATERIAL AND METHODS.

The material consisted of winter ovotestes of *Helix aspersa*. In these there were comparatively few mitoses, but we did find spermatids and all other stages. The gonads were ultra-centrifuged at about 50,000 times gravity for varying periods up to ten minutes. Two to five minutes were quite long enough to produce an effect. We examined the material both fresh and fixed. Figs. 1-6, Pl. 19, were drawn from fixed<sup>1</sup> cells, but they apply equally well to living spermatocytes in which the layers produced by centrifugation were visible. Controls were used, though somewhat unnecessarily, as leaving pieces of ovotestes for ten minutes in Ringer does not separate the cytoplasmic inclusions into layers.

#### DESCRIPTIONS OF RESULTS.

The normal *Helix* spermatocyte is a well-known cytological object, having been studied by a large number of workers from the time of v. La Valette St. George in 1867. A modern account of these cells is found in the second part of the 'Cytoplasmic Inclusions of the Germ Cells', by Gatenby (9), and the behaviour of these cells to neutral red in a paper by Douglas, Duthie, and Gatenby (7). In fig. 1, Pl. 19, is a typical spermatocyte. The Golgi apparatus, which is visible intra-vitam without staining,

<sup>1</sup> Champy's fluid, iron haematoxylin.



is formed of batonettes (not scale-like bodies) embedded in the surface of the archoplasm. During mitosis some of the batonettes or Golgi bodies break up into smaller pieces becoming detached from the archoplasm, and all the batonettes (dictyosomes) tend to keep around the asters and are so divided into two subequal groups in each daughter cell.

In fig. 5, Pl. 19, is a typical centrifuged spermatocyte showing two layers, the upper occupied by the Golgi apparatus, the lower by the mitochondria, clear cytoplasm (cc.) being in between. The nucleolus has not been moved. In figs. 2 and 4 are two other spermatocytes, but these show a clear uppermost space or vacuole. In fig. 2 the space is just above the Golgi apparatus, whereas in fig. 4 the Golgi apparatus lies to one side, centripetally to the mitochondria. In fig. 6 the vacuole (sr.) is larger, the cell more elongated, and the nucleus necrotic. The Golgi apparatus is still uppermost under the vacuole.

In fig. 3, Pl. 19, is a cell at the prophase of division, and it is one of a few cases we have found of the centrifuged cells undergoing mitosis. The nucleolus (NL.) has passed through the nuclear membrane (which must be weak at this stage of mitosis), the chromosomes are still within the nuclear membrane but have passed centrifugally, the mitochondria, which are very filamentous at this stage of mitosis, have formed a distinct group, and above on each side are separate groups of Golgi elements (GA.). Such cells are of special interest because the Golgi elements are free from the archoplasm, but, even so, are lighter than the mitochondria.

In some of our slides all the spermatocytes in certain diverticula of the ovotestis have broken down, but their former confines are marked by lightly stained cell walls. In these ghost-like cells the only surviving inclusions are the Golgi elements. These appear to be the least fragile part of the cell.

In some of the ultra-centrifuged cells the Golgi elements are found among the mitochondria at the centrifugal pole. Such conditions are, we think, explained by the fact that the Golgi elements have been trapped by the centrifugally dispersed nucleus and mitochondria preventing them from taking up their usual position as shown in figs. 2-6, Pl. 19.

## DISCUSSION.

Spermatocytes of *Helix aspersa* contain two categories of cytoplasmic bodies, Golgi bodies and mitochondria. The latter are comparatively heavy, as shown previously by Fauré-Fremiet (8), Browne (1), Gatenby (10), Brambell (4), Harvey (15), Beams and King (1-2 a). The dictyosomes or Golgi bodies are somewhat lighter and rise up above the mitochondria when the cells are centrifuged; so that the two categories are separate. This separation is not due to the presence of archoplasm, because it holds good in cells undergoing mitosis in which the Golgi apparatus has broken up into its several parts.

Ultra-centrifuged spermatocytes are readily injured if left too long, or centrifuged too rapidly, as shown by the presence of a watery vacuole which soon implicates the whole cell. In cells which have broken down to an unstainable matrix in the diverticula of the ovotestis, the cell walls and, much more so, the Golgi elements are still unchanged. The survival of the latter during the final disintegration of the cell is an interesting phenomenon, and shows how different in formation they must be from mitochondria.

The ultra-centrifuging of the cell has provided us with some extremely interesting information regarding the relative consistency and relative specific gravity of the Golgi apparatus and mitochondria. In the case of the guinea-pig uterine gland cells, the Golgi material is apparently comparatively liquid enough to form spheres when the streamers break up during centrifuging (Text-fig. 4). Moreover these spheres do not mix with the cytoplasm.

It is noteworthy that the Golgi material of *Helix* spermatocytes, uterine gland-cells of *Cavia*, and the osmiophilic platelets of the bean root-tip cells, are all in general lighter than the surrounding cytoplasm, and move to the centripetal pole, while the mitochondria are heavier than the cytoplasm and collect at the centrifugal pole.

In the recent discussion on the Golgi apparatus at the Second International Cytological Congress, at Cambridge, a majority of workers stated that no such structure as the Golgi apparatus

existed, but that it was a neo-formation produced by fixation. It will be apparent from the facts brought forward in this paper that such a view is now untenable.

The microchemical evidence such as can be got by fixation and staining has shown that the Golgi material and mitochondria are not quite the same, and now the ultra-centrifuge has added to this evidence by showing that the Golgi apparatus of the germ and somatic cells is much lighter than the mitochondria and not mixable with the cytoplasm.

### SUMMARY.

In the spermatocyte of *Helix*, as in the guinea-pig uterine gland cells and in the root-tip cells of the bean, the Golgi bodies pass centripetally whereas the mitochondria pass centrifugally when 'ultra-centrifuged'.

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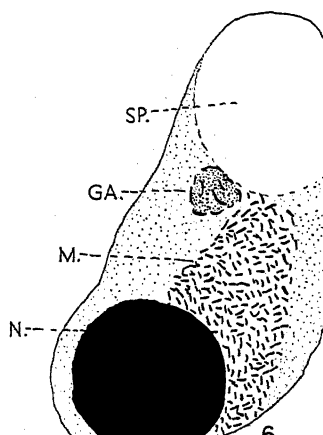
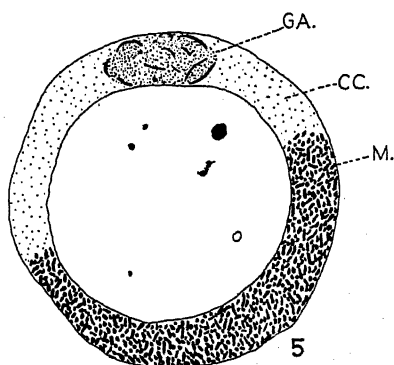
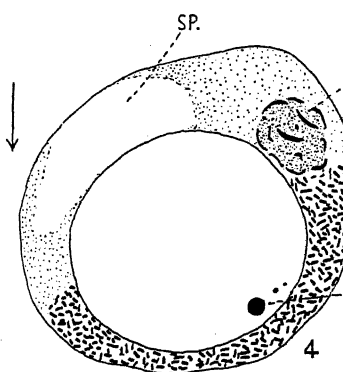
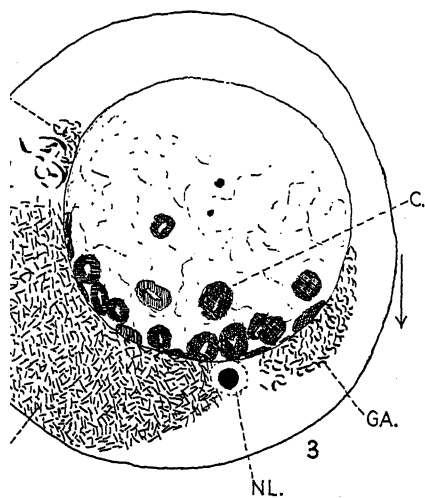
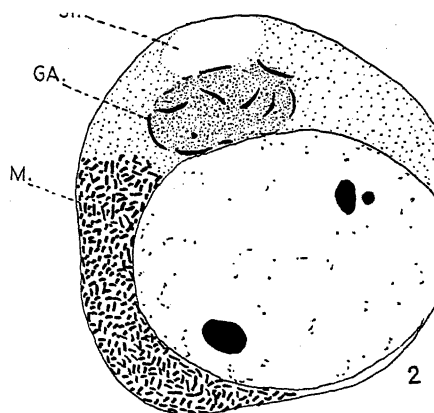
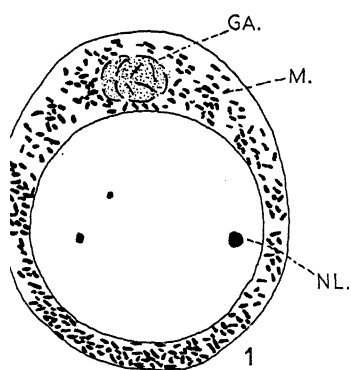
## EXPLANATION OF PLATE 19.

### LETTERING.

*C.*, chromosome; *C.C.*, clear cytoplasm; *G.A.*, Golgi apparatus; *M.*, mitochondria; *N.*, nucleus; *N.L.*, nucleolus; *S.P.*, watery space.

All figures drawn from preparations by Champy iron alum haematoxylin. Fig. 1. Normal spermatocyte; Figs. 2-6. Ultra-centrifuged spermatocytes.







# Studies on the Cytoplasmic Components in Fertilization

## I. *Ascaris suilla*

By

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With 9 Text-figures.

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THIS study of the cytoplasmic components was done on the fertilized eggs of *Ascaris suilla*. This species of *Ascaris* is found in the intestinal tract of the common domestic hog and is identical morphologically with *Ascaris lumbricoides*, the roundworm found in man. It has been proved conclusively by several of the Japanese workers that although *Ascaris suilla* is morphologically identical with *Ascaris lumbricoides*, it is physiologically a distinct species in that *Ascaris suilla* will not live in the medium of the human intestine, and *Ascaris lumbricoides* will not live and grow in the intestine of the hog.

The worms used were taken from the intestines of freshly slaughtered hogs at the abattoir (incidentally, the infection of hogs with *Ascaris suilla* is very high), placed in warm normal saline, and taken to the laboratory. The uteri and the ovaries were immediately dissected out in fresh warm normal saline and placed in the killing fluids. Most of the material was run through as serial sections so that the exact location in the uterus or ovary could be determined. This required painstaking work as each small piece of tissue was run separately through the entire schedule to the finished slide.

An incidental study was made of the formation of the sperm so as to determine the exact formation of the refringent body or acrosome. It is interesting to note here that it was impossible to find fully matured sperm in the male. They were found only in the uterus of the female. Careful measurements were made



of the acrosome so that the volume of refringent bodies in the fertilized eggs could be compared.

Practically all techniques were used with varying degrees of success. All the preparations involving the use of osmic acid were eminently successful, but the silver preparations were poor and entirely useless for accurate observations. While the osmic preparations were exceptionally good, the final choice for demonstrating the Golgi material was Kolatchev's method as outlined by Bowen (1928). This schedule was modified slightly to lower the temperature and shorten the length of osmication, and this resulted in extremely delicate and fine impregnations.

Greater difficulty was experienced in satisfactorily demonstrating the mitochondria, but finally slight variations in the techniques of Benda and Champy-Kull were employed. The final results with mitochondria were very good.

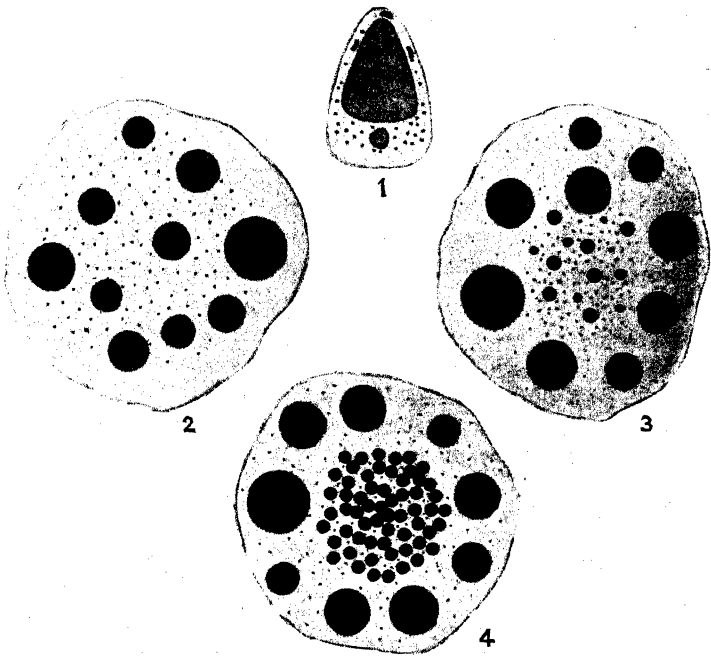
Considerable work was done in the counterstaining of the osmic impregnated sections, and some very valuable results which threw light on the entire problem were obtained.

At this point I shall briefly summarize my observations and then later go into a detailed discussion of these and attempt to clarify the whole problem in the light of the work done up to the present.

The sperm fertilizes the egg, not in the uterus, but well up in the oviducts. As soon as the sperm enters the egg a delicate fertilization membrane is thrown off, preventing normally the entrance of other sperm. After a short time the sperm disintegrates and the nucleus undergoes its well-known routine. However, the other cytoplasmic components, in which we are particularly interested, undergo a routine quite as definite as that of the nucleus. These cytoplasmic components are of three kinds: the mitochondria, the Golgi bodies, and the refringent bodies. From this point it is advisable to treat each of these separately.

### 1. Mitochondria.

As soon as the sperm has disintegrated the paternal mitochondria become scattered in the cytoplasm of the egg where they are indistinguishable from the maternal mitochondria



TEXT-FIGS. 1-4.

Fig. 1.—Slide 11032-7. Position 38.3-99.1. This is a mature sperm. The larger black bodies are remnants of the Golgi bodies which were not sloughed off. The amount of Golgi material remaining in the sperm is very variable. Usually there is hardly an appreciable amount remaining. The small black granules are mitochondria.

Fig. 2.—Slide 112131-9. Position 35.6-96.9. The refringent bodies have been formed by the breaking down of the acrosome of the sperm and are stained intensely red by acid fuchsin. The Golgi bodies and mitochondria are impregnated black and are practically indistinguishable from one another.

Fig. 3.—Slide E22632-17. Position 45.0-97.9. The Golgi bodies have begun to enlarge and are impregnated a deep black by osmic acid. There are no changes in the refringent bodies or mitochondria.

Fig. 4.—Slide C12132B-12. Position 42.3-99.5. The Golgi bodies have still further increased in size until they crowd the central portion of the egg. The refringent bodies have completed their migration to the periphery of the cell.

(Text-fig. 2). They are present in these earlier stages as very minute round granules, uniformly distributed throughout the cytoplasm. There is no particular grouping of these bodies in any specific portion of the cell. They are impregnated a deep black by osmic acid but can be bleached rapidly in hydrogen peroxide and slowly—but selectively—in oil of turpentine, which bleaches out the mitochondria but does not appreciably affect the Golgi bodies. After bleaching, the mitochondria may be counterstained with either acid fuchsin or crystal violet. As the cell develops they increase greatly in number, although they remain constant as to size and shape (Text-fig. 4). As the egg continues to develop the mitochondria become reduced in number, by some process which I have so far been unable to determine, until in the mature egg they are present as a small number of minute granules (Text-fig. 9).

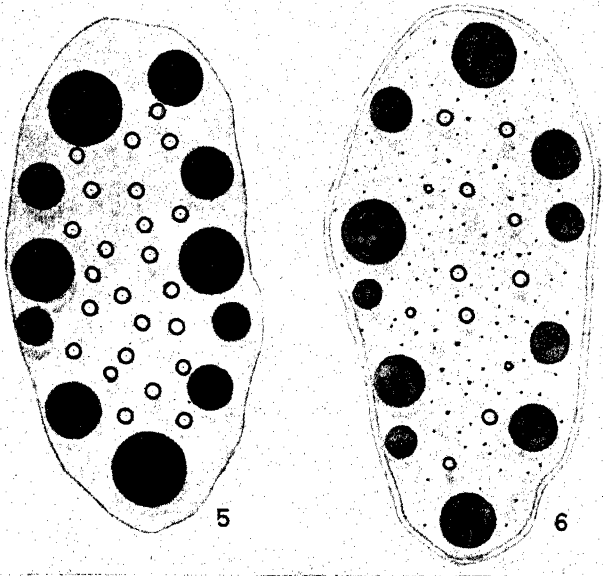
## 2. Golgi Bodies.

There seems to be no Golgi material brought into the egg by the sperm (see explanation of Text-fig. 1). The form of these bodies in the egg is identical with that of the mitochondria, and the two can be differentiated only by careful bleaching in oil of turpentine. At about the time the refringent bodies orient to the periphery, the Golgi bodies begin to increase in size and number until they almost completely fill the central portion of the cell (Text-figs. 3 and 4). At this time it is possible, by careful bleaching with hydrogen peroxide, to differentiate these bodies into an outer osmiophilic ring and an inner osmiophobic portion (Text-fig. 5). It is possible also, at this stage, to bleach the Golgi bodies completely and counterstain them with crystal violet. The mitochondria will take the crystal violet but not to so great a degree as the Golgi bodies. As the cell-wall thickens and the refringent bodies shrink, the Golgi bodies become reduced in size until they regain their original appearance, small black granules scattered uniformly throughout the cytoplasm (Text-figs. 8 and 9).

## 3. Refringent Bodies.

The acrosome enters the egg, breaks down, and forms a num-

ber of round bodies which become scattered throughout the cell (Text-fig. 2). These bodies gradually become larger through some process which is not apparent, although it seems to be by

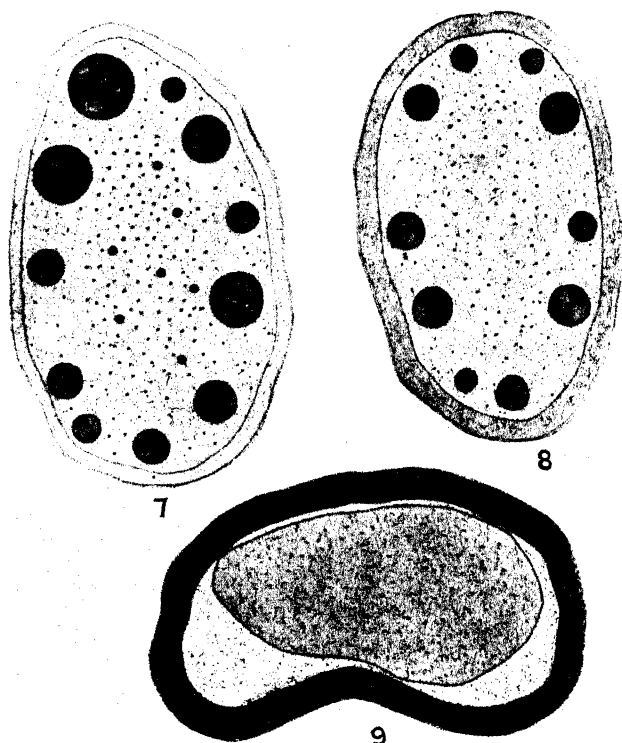


TEXT-FIGS. 5-6.

Fig. 5.—Slide 41832-23. Position 56.6-101.8. This egg, which is at a stage slightly later than Text-fig. 4, shows the selective bleaching out of the Golgi bodies to show their dual nature. The mitochondria are bleached out, leaving only Golgi bodies. The eggs at this time have assumed the shape of a prolate spheroid, characteristic of the more mature eggs.

Fig. 6.—Slide 42532-2. Position 51.0-104.1. This egg is similar to Text-fig. 5, but the refringent bodies are showing their first signs of vacuolation. The heavy cell-wall has just appeared.

absorption from the surrounding cytoplasm. The refringent bodies are moderately osmiophilic and decidedly fuchsinophilic in their staining reaction. They are, at this time, large and perfectly round homogeneous masses of varying sizes. They soon begin to migrate and arrange themselves around the periphery of the cell (Text-fig. 4). In a short time they undergo an internal



TEXT-FIGS. 7-9.

Fig. 7.—Slide C12132A-7. Position 65.6-108.7. Here the vacuolation of the refringent bodies is well under way and the heavy cell-wall is beginning to thicken and to acquire the fuchsinophilic staining reaction. The Golgi bodies have become smaller. This egg has just passed the point of maximum cellular activity.

Fig. 8.—Slide 122032-31. Position 56.1-106.5. The cell-wall has almost attained its maximum thickness and is definitely displaying its affinity for acid fuchsin. The refringent bodies are almost entirely consumed. The Golgi bodies have shrunk to their original size.

Fig. 9.—Slide 31732-62. Position 52.5-105.5. The cell-wall has attained its maximum thickness and affinity for fuchsin. The refringent bodies are entirely consumed. The Golgi bodies are now indistinguishable from the mitochondria. In this stage, the cytoplasm has shrunk back from the cell-wall and we have the typical fertilized egg of *Ascaris* as ordinarily seen.

vacuolation. The cell-wall then begins to thicken, and as it increases in size the refringent bodies become reduced. As they gradually shrink in size they lose their affinity for acid fuchsin. Simultaneously, the cell-wall begins to progressively acquire it. Finally when the refringent bodies have been entirely used up the cell-wall has become extremely heavy and decidedly fuchsinophile.

TABLE I.

Approximate Physical Measurements of *Ascaris suilla* Sperm and Eggs.

Acrosome	Length	0.0126 mm.
	Diameter (at base)	0.0084 mm.
	Total volume	$2.2 \times 10^{-9}$ cu. mm.
Sperm	Length	0.0175 mm.
	Diameter (at base)	0.0105 mm.
	Total volume	$7.3 \times 10^{-9}$ cu. mm.
Round eggs (these are freshly fertilized)	Diameter	0.042 mm.
	Total volume	$4.9 \times 10^{-5}$ cu. mm.
Ovoid eggs (these are approaching maturity)	Diameter—Major Axis	0.063 mm.
	Diameter—Minor Axis	0.035 mm.
	Total volume	$3.75 \times 10^{-5}$ cu. mm.
Miscellaneous	Approximate total volume of refringent bodies	$8.535 \times 10^{-7}$ cu. mm.
	Volume occupied by mature cell-wall	$0.83 \times 10^{-5}$ cu. mm.

These figures in this table are based on numerous measurements and represent in each case the averages of that particular series of measurements.

The acrosome occupies a little less than one-third of the total volume of the sperm. The egg is approximately six thousand seven hundred times as large as the sperm. The more mature ovoid eggs are only 76 per cent. as large as the freshly fertilized round eggs. In a young egg, the refringent bodies occupy about one-fiftieth of the total volume of the egg. In mature eggs the cell-wall occupies about 20 per cent. of the total egg volume.

Upon first consideration these figures may seem rather astounding, but after careful study and checking one comes to the conclusion that they are not out of line with what one would suspect after a careful study of any type of egg during fertilization.

TABLE II.  
Comparison of Volume Increases.

Volume of Acrosome. . . . .	0.0000000022 cu. mm.
Total volume of Refrangent Bodies . . . . .	0.0000008535 cu. mm.
Volume occupied by Cell-wall . . . . .	0.0000083 cu. mm.

From this we can see that, in their formation from the acrosome, the refrangent bodies have increased in size about three hundred and eighty-eight times. The mature cell-wall occupies about nine times the volume of the refrangent bodies from which it was formed.

### DISCUSSION.

I have been unable to demonstrate any signs of a remnant of the original Golgi material in connexion with the acrosome in spite of numerous and varied attempts to accomplish this specific fact. There are, however, usually a few fragments remaining in the cytoplasm after the bulk of it has been cast off. I attach no significance to these and attribute their presence to a mere mechanical fault in the sloughing off of the majority of the Golgi material.

I am convinced, after considerable study, that the refrangent body of the sperm is actually the acrosome. It is homologous with the acrosome of flagellate sperm, not by virtue of its position in the sperm, but because it follows the established and invariable rule for the formation of acrosomal material from the Golgi complex (in this I am in accord with Bowen (1925)).

The refrangent granules arise in the formation of the sperm as a differentiation product of the Golgi material in a manner comparable to the formation of the acrosome in flagellate sperm. As the granules are formed, their connexion with the Golgi bodies is severed and they collect in the cytoplasm in large numbers. Any relationship which they may seem to have with the mitochondria is purely topographical. During the spermatid stage these numerous bodies come together and fuse to form one large refrangent body, or, as I shall term it, an acrosome.

The acrosome of the sperm breaks down after fertilization and forms a variable number of perfectly round bodies. These bodies soon become several times larger. This process of enlargement is through absorption of fluids from the cytoplasm. These bodies are incapable of actual growth. This fact is further

borne out by the lack of Golgi material in the acrosome. Their enlargement is comparable to a swelling. The actual amount of lipoidal material present remains constant, if its affinity for fuchsin is any criterion. When small the bodies are intensely fuchsinophile. As they grow larger the shade gradually becomes lighter, although it remains a brilliant homogeneous red. The total volume of the enlarged refringent bodies as shown in Table I is approximately three hundred and eighty-eight times that of the acrosome. This factor is fairly constant as determined by numerous measurements and computations.

These refringent bodies, lipoidal secretion products of the Golgi complex, are utilized in the formation of the heavy cell-wall which is characteristic of fertilized *Ascaris* eggs. This fact can be demonstrated by their orientation and shrinkage. As these bodies become vacuolated the cell-wall begins to thicken and progressively acquires the fuchsinophile staining reaction formerly exhibited by the refringent bodies. Finally the bodies are fully consumed and the cell-wall has attained its maximum thickness.

Another point which tends to prove my contention that the refringent bodies are concerned in the formation of the heavy cellular wall is that only those eggs which are fertilized develop this wall. This fact has long been known and is even employed as a diagnostic feature of fertilized *Ascaris* eggs by clinical pathologists. Unfertilized eggs cannot form a wall as they do not possess the material which is supplied by the acrosome of the sperm.

This idea of the function is not new; although, to my knowledge, it has not been worked out. Quoting from Bowen (1925), we find:

'With these uncertainties still to be resolved, it is perhaps hazardous to venture on a discussion of the function of the acrosome based on the conditions in *Ascaris*. But I cannot refrain, in concluding, from calling attention to the fact that, coincident with the dissolution of the refringent body, very extensive changes occur in the cytoplasm of the egg. These eventuate in the extrusion of a considerable amount of material from the egg, which forms a shell that encloses the egg in one



of the most remarkable membranes of its kind. That there is some relation between the extensive cytoplasmic activity which produces this unusual membrane, on the one hand, and the remarkably large quantity of "acrosomal" material, on the other, is an hypothesis too attractive to be avoided and in such a possibility I find the basis for some tangible evidence bearing on the function of the acrosome. In any event, if these suggestions prove acceptable, we have in the *Ascaris* sperm a remarkable possibility for critical experimental attacks on the microchemistry of the acrosome.'

Meves (1911) also observed the behaviour of the refringent body in the egg of *Ascaris*, although he did not attach this particular significance to it. He described it but did not picture its breaking down. Meves's conclusion was that the refringent body was gradually dissolved and its substance liberated into the cytoplasm.

I think in this form we may definitely throw out Bowen's (1924a) suggestion that the 'acrosome is an enzyme (or a complex of enzymes) comparable to other secretory products, the primary function of which is to set off the fertilization reaction'. This would second Lillie's fertilizing theory. This is an excellent hypothesis, but is not supported by the findings in *Ascaris*.

Marcus (1906) considers the refringent body as yolk which serves as a nutritive material for the sperm. This may possibly be true up to the point at which the several bodies fuse in the spermatid to form the acrosome, but beyond that point it is impossible because there is no using up of its substance until after it disintegrates in the egg to form the refringent bodies.

Scheben's (1905) conclusion that it is the achromatic structure of the nucleus, and Romieu's (1911) that it is a protection for the nucleus and a fixed point in the ameboid movement supposed by him to be attendant upon the efforts of the sperm to penetrate the egg, are so impossible as to require no further comment here.

As to the mitochondria, one cannot be so positive. It appears that they must be vitally concerned in some way with the cellular metabolism, as they are most numerous and active

when cellular activity is greatest, and are quiescent and few in number when the cell is dormant.

'According to many students of the mitochondria, dating from the pioneer researches of Altmann, these bodies are directly transformed into secretory granules. Others see in the mitochondria only synthetic centres where the raw secretory materials are built into the definitive granules. In spite of these divergent accounts, Duesberg (1911) concludes that the connexion of the mitochondria with secretory processes is proved, while Cowdry (1918) seems inclined to draw an exactly opposite conclusion from practically the same data. On this point I am in accord with Cowdry and cannot agree with Duesberg that, in spite of fundamental differences of fact, the agreement of the mitochondrial workers as to the final outcome is sufficient to establish a probability in their favour. Rather does it seem to me that these differences clearly show a gap in the evidence which is so decided that different workers find in the same material fundamental morphological differences. In view, therefore, of the conflicting statements of fact and the known sources of error, it appears probable to me that the origin of secretory granules from mitochondria, ergastoplasm, and related cytoplasmic elements must be looked upon as unproved and of doubtful utility as a working hypothesis' (Bowen, 1924).

I have particularly attempted to check up, by observation only, on Wallin's (1922 et seq.) theory that the mitochondria are possibly symbiotic organisms in the cytoplasm, but to date I have found no facts which would, in my mind, even remotely suggest this possibility.

I can find no evidence which would tend to support Meves's findings in *Ascaris megalocephala*. Meves (1911) advanced the idea that the mitochondria were possibly concerned in heredity. He states that it is possible to follow the maternal and paternal mitochondria after fertilization and to differentiate between the two types. He even goes so far as to figure pairings of the maternal with the paternal mitochondria. I find it absolutely impossible to distinguish between these mitochondria once they become intermingled in the cell. While their function

is still extremely doubtful, I feel it is safe to rule out this suggestion of Meves.

The Golgi bodies present by far the most interesting and instructive figures in these cells.

There is very little if any true Golgi material brought into the egg by the sperm, so we may be sure that most of the Golgi material observed is maternal in origin.

After osmic impregnation the Golgi bodies appear first as small, black granules which are distinguishable from the mitochondria only after careful differentiation in oil of turpentine, which bleaches out the mitochondria and does not appreciably affect the Golgi bodies. As the cell becomes more active these Golgi bodies, after a numerical multiplication, increase in size by an inward secretion which fills their interior with a fat which is selectively bleached out in oil of turpentine without injury to the impregnation of the Golgi material proper. This leaves the true Golgi apparatus as an osmiophilic outer ring, or rather sphere.

This type is similar to that found by Vishwa Nath (1924 et seq.) in his studies of oogenesis. Nath found that one kind of yolk material is formed by an apparently direct transformation of the Golgi substance itself. I fail to find any evidence which would tend to postulate a direct transformation theory, but rather imposing evidence that the Golgi material is an intermediary or synthesizing centre for yolk formation.

Ludford (1921) describes a development of yolk-spheres in association with the Golgi apparatus essentially comparable to the mode of origin of the acrosome and to the formation of the yolk-spheres of *Ascaris*.

Gatenby and Woodger (1920) reviewing vitellogenesis find numerous and entirely different methods, one of which is by the Golgi bodies.

Hirschler (1918) pointed out that, in the case of hollow spheres, the central contents could not possibly be the same as the external cytoplasm, and thus could be considered as a part of the Golgi apparatus. To this central structure, which he never succeeded in staining differentially, he gave the name 'apparatinhalt'. I, however, have succeeded in differentially

staining this inner substance, and I am convinced that it is of a fatty-lipoidal nature. Hirschler's observations were made on the early embryology of another form, so the case is comparable to the one under consideration.

Nassonov (1922) figures a close positional relationship between the strands of Golgi material and the smallest visible secretory granules. The granules subsequently lose their connexion with the Golgi material. The growth of the granules is not, however, confined to their period of association with the Golgi material, but is continued independently. Associated, however, with such granules there is usually a girdle of material which impregnates like the Golgi apparatus and which Nassonov interprets as a piece of Golgi material which the granule has carried away when its original connexion with the Golgi apparatus is severed. Nassonov does not give a final interpretation to these observations, but he does make three possible suggestions. I quote these from Bowen (1924 a).

'First, one might explain the secretory products as a direct transformation of the Golgi material itself—a view which the facts do not give any definite support. In the second place, the Golgi reticulum may play merely the role of an intermediary between the ultimate secretory granules and some other source which furnishes the raw material. These raw materials might be mitochondrial granules, which are transformed into secretory granules proper through the influence of the Golgi apparatus; or they might come from the undifferentiated cytoplasm out of which, in immediate contact with the meshes of the Golgi network and under the influence of the organoid, the granules are synthesized.'

This last suggestion is the one nearest to the picture. I find that these 'raw materials' come from the undifferentiated cytoplasm rather than from the mitochondrial granules.

In conclusion on this point I may say that in *Ascaris* sperm and eggs all secretory granules when first visible are found in intimate connexion with the Golgi apparatus, and usually this association is for a considerable length of time.

I am also convinced that these Golgi bodies indisputably

function to secrete yolk-fat which is later utilized in intracellular metabolism.

It is quite evident to me that the role of Golgi bodies in these cells is to secrete fatty-yolk. This fact can be quite definitely demonstrated by selective bleaching of the enlarged Golgi bodies to show their dual nature. It can be proved that oil of turpentine will selectively bleach fat.

The central portion of these bodies is dissolved out in a short time by the oil, but the outer osmiophilic rim is unaffected even after exposure to the oil for several months.

This fat is utilized in cellular metabolism. The Golgi bodies, small granules at first, become filled with fat by internal secretion just before the cell arrives at its period of maximum activity. During this period the cell absorbs this fatty substance from the Golgi bodies and uses it in its physiological functioning until in the end the Golgi bodies have been reduced to small granules only slightly larger than the mitochondria. This type of secretion is similar to that found by Vishwa Nath and his co-workers in their studies of yolk-formation.

Bowen describes two types of yolk in the fertilized egg of *Ascaris*. One of these is of a lipid nature and is easily blackened by osmic acid. It forms the familiar oil cap at the centripetal pole of a centrifuged egg. This, I find, is the true yolk of the egg. The other type of 'yolk' is usually more abundant and does not ordinarily blacken in osmic acid. It occupies the heavy pole of a centrifuged egg. Bowen believes the latter represents what is in itself ordinarily termed 'yolk', but he is absolutely wrong. This substance is a conglomeration formed from the numerous refringent bodies and has no connexion with any type of yolk.

The Golgi bodies and the mitochondria, while not being morphologically or genetically related, are certainly very similar in their chemical nature. Their reactions to osmic acid and other reagents are comparable although not identical. Their physiological behaviours also have much in common. They are both concerned in cellular metabolism; one as a synthesizing centre, and the other as more of a regulating or control centre. This control over cellular activity by the mitochondria seems to be

in the nature of an enzymic or catalytic action. Its mechanism is not apparent, but its effect is clearly noticeable.

Quoting Bowen (1926 a), 'The Golgi apparatus would appear to be the synthetic centre wherein are built up primarily those cellular products which in the broadest sense of the term may be classified as secretions.'

I disagree most emphatically with those workers who contend that the Golgi bodies are artefacts and the results of fixation and impregnation. Walker and Allen (1926) state that the same results may be obtained with certain colloids containing lecithin and kephalin. In my estimation this merely would prove that the Golgi bodies are probably colloids containing lecithin and kephalin, rather than that they are merely artefacts. A matter of interpretation, of course, but the facts seem rather against their theory.

It is impossible that these particular Golgi bodies are artefacts. Their occurrence and appearance after a variety of techniques is too definite and orderly to be the result of chance, as those who upheld the artefact theory would have us believe.

My results tend to corroborate those of Covell and Scott (1928). They found that, in nerve-cells, the granules stainable with neutral red in the living cell, upon treatment with osmic acid, come together and fuse to give a reticular or strand-like formation to the Golgi material. I find this occurs in these eggs, but only after rather prolonged osmication, particularly at higher temperatures (35°–40° C.). It was possible, by carefully controlling the osmication and by using lower temperatures (25°–30° C.), to secure extremely delicate impregnations.

After careful consideration I am convinced that a reticular structure of the Golgi apparatus, in this type of cell at least, is a result of technique rather than a true picture of the living cell.

It was impossible to do as much intra-vitam work along these lines as I would like to have done. It is extremely difficult to get the dye to penetrate the heavy cellular membrane of *Ascaris* eggs, and the only satisfactory method of carrying on this type of experiment would be with a micro-dissection apparatus, which was not available.

My conclusion on this point is that in the fertilized eggs of

*Ascaris suilla* the normal shape of the Golgi bodies is always spherical, and variations are entirely due to technical difficulties.

I can find no evidence which would support either Holmgren's trophospongium theory or the canalicular theory. These types simply do not exist in *Ascaris* eggs.

Although I had originally planned to continue these studies on other forms, I have found such widely divergent descriptions of spermiogenesis and fertilization in the various papers on *Ascaris megalocephala* that I have already begun an exhaustive study of these phenomena in this form. I have decided to devote the second paper of this series to a detailed discussion of my own findings in *Ascaris megalocephala* and attempt to dispel the confusion in which the literature on this subject leaves one. In particular do I wish to discuss in detail Hans Held's important paper on *Ascaris megalocephala* (1917).

#### CONCLUSIONS.

1. The so-called refringent body of the *Ascaris* sperm is actually an acrosome.
2. The function of the acrosome is to supply the material from which the heavy cell-wall is formed.
3. The acrosome does not appear to have any actual connexion with the process of fertilization, as suggested by Bowen, Lillie, and others.
4. The mitochondria seem to function as a regulating or control mechanism in cellular metabolism, and are apparently not concerned in any secretory activity unless it is of an enzymic or catalytic nature.
5. The growth of the Golgi bodies is by the inward secretion of a fatty substance. The true Golgi material is an outer osmophilic sphere. The substance secreted fills the interior of the enlarged Golgi bodies.
6. The form of the Golgi bodies is always spherical. They appear as small granules when the cell is quiescent and enlarge as the cellular metabolic rate increases. Normally there is never a reticular structure.

7. The secretory product of the Golgi bodies is utilized in cellular metabolism.

8. All visible secretory products in *Ascaris* arise in connexion with the Golgi bodies.

9. The Golgi bodies are definitely not artefacts.

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# The Sperms of the British Muridae

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With 30 Text-figs.

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## I. INTRODUCTION.

THE Muridae are first known from the Lower Eocene and since then have evolved to become the largest of the Mammalian families. When, therefore, Dr. J. R. Baker suggested to me that a survey of the spermatozoa of a restricted group of animals would be of general zoological and evolutionary interest, I agreed to undertake the work and had no hesitation in choosing the Muridae as the group to work upon. They seemed to be excellent material for this purpose, for their successful evolution means that, even in a small area like Great Britain, several genera and species are accessible and reasonably plentiful. Further, the distinctive sperm of the Muridae gave additional interest to the group.

The work was started in the Department of Zoology and Comparative Anatomy at Oxford, and I thank Professor E. S. Goodrich and Dr. J. R. Baker for their encouragement and help. Mr. C. Elton and his staff of the Bureau of Animal Population were always ready with advice and practical help in field-work,

and I am very grateful to them. Later the work was continued in Edinburgh, and I am much indebted to Professor J. H. Ashworth for reading the manuscript, for his stimulating interest, and the facilities I have enjoyed in the Department of Zoology here.

Part of the cost of the work was defrayed by grants from the Christopher Welch Trust and the Earl of Moray Fund.

Although most of the animals examined were trapped by myself, specimens of three species I failed to get were sent to me by friends. I am acknowledging this great service in the appropriate place, but I am much indebted to Mr. A. R. Thompson for putting me in touch with two of these helpers.

## II. GENERAL METHODS.

Individuals of the species required were obtained and the males kept until convenient. One was then killed suddenly and the epididymes were quickly dissected out and transferred to 0.9 per cent. saline. In this fluid the tubules were cut once or twice and gently squeezed between the forceps to hasten the issue of the sperms. The resulting suspension of sperms was examined under the microscope and used only if it came up to the usual standard of density and motility.

If the suspension passed this simple test some sperms were mounted, unfixed and unstained, in saline and sealed; while to other similar fluid mounts was added a drop of gentian violet which killed, fixed, and stained the sperms and provided a semi-permanent preparation. Other portions of the same suspension of sperms were fixed, some in the vapour of 2 per cent. osmium tetroxide for ten minutes and others in a one-tenth standard sublimate-acetic solution for thirty minutes. The osmium-fixed sperms were later fixed to the slide and stained either in 1 per cent. gentian violet, 1 per cent. basic fuchsin (Rosanilin), or Heidenhain's haematoxylin and mounted either in a saturated solution of potassium acetate or in balsam. The fluid mounts were sealed with Rousselet's cement and gold size and proved to be reasonably permanent. The sperms which were fixed in sublimate-acetic solution were stained, some in haematoxylin and some by Feulgen's technique, with light green as a counter-

stain. When time and material allowed other preparations by different methods were added to this standard series.

### III. METHODS OF MEASUREMENT.

At an early stage in the work it was realized that microscopic examination alone was not sufficient and would have to be supplemented by measurement. The various methods for measuring small objects were tried, but finally it was decided to use a screw-micrometer with a Ramsden eyepiece. This choice depended largely on personal preference, but the screw-micrometer, once calibrated, had the advantage of giving immediately a direct measurement, correct to the nearest quarter micron.

The use of the screw-micrometer was extended beyond simple measurements of lengths to give scale drawings of the sperm-heads by means of Cartesian co-ordinates. This involved passing the travelling hair line of the micrometer over the image of the sperm in one direction and following this traverse by another at right angles to it. As it passed, micrometer readings of the prominent features of the sperm were taken and later their co-ordinates were plotted on squared paper. One of the rectangular axes of the co-ordinates was orientated parallel to a datum line on the sperm-head which had previously been decided upon for each species. It was desirable that such a datum should be parallel to the main normal axis of the sperm. Thus for the house mouse the posterior straight part of the concave side of the head served for the ordinate and a line at right angles to this for the abscissa. Once the main outline had been blocked in on the squared paper finer details could be added, using a higher powered ocular. In this way, which may seem complicated, but which was in practice quite simple, most of the accompanying figures were drawn.

A series of measurements, designed to get some idea of the effect of various techniques on the head-lengths of sperms was carried out on a complete set of preparations from the house mouse. These showed that sperms mounted in potassium acetate, after being fixed in osmium vapour and stained in gentian violet, gave the closest approximation to the size of



those mounted in saline. This method, therefore, since it also provides stained mounts which can be measured with far less eye strain than is involved in measuring fresh sperms, has been used in this work as a standard technique for all comparative measurements. The mean for saline mounted sperms of the mouse was  $8.09 \pm 0.04 \mu$ , the standard deviation (S.D.) being  $0.28 \mu$ , and that for standard technique  $8.27 \pm 0.04 \mu$ , S.D. being  $0.31 \mu$ , the latter method causing a swelling of 2 per cent., approximately, which compares favourably with a shrinkage of 6 per cent. for sublimate, haematoxylin, and balsam preparations.

#### IV. NOMENCLATURE.

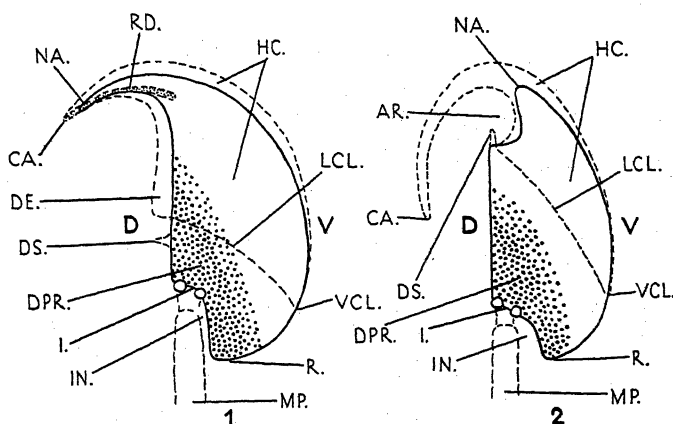
There is no standard nomenclature for the finer points of the morphology of these spermatozoa. Parat (12) has recently attempted one for certain cytoplasmic elements, but his type sperm is so generalized that his names are of little value for the present purpose.

Text-figs. 1 and 2 are diagrams of the two main types of sperm-head I have found in the Muridae and they are labelled according to the scheme I am using in this paper. The conventions 'dorsal' and 'ventral' are those used by Retzius (15). The term 'head-cap' I have chosen from several alternatives to denote all the anterior extra-nuclear part of the head of the sperm. The usual name 'acrosome', because it has been used for the idiosomal part only of this region, and the term 'perforatorium', with its functional meaning, are both rejected in favour of one which is simple and descriptive. The term 'dense posterior region' is used to describe that asymmetrical deeply staining area which is found in the posterior part of the nucleus in all the Muride sperms I have seen. In position it agrees with Gatenby's post-nuclear body (6, 7), but since it is intensely stained in Feulgen preparations and I know nothing of its origin I have not felt justified in assuming it to be a post-nuclear body in the strict sense.

#### V. THE MURIDE SPERM.

Most of the sperms of the Muridae are of a highly specialized type which is easily recognized by its flattened hook-shaped

head. For this and other reasons Retzius (15), to whom we are indebted for the widest survey of animal sperms yet published, places them in his fourth and most specialized of the groups into which he divides the sperms of the Rodentia. The essentials of his classification are as follows:



TEXT-FIGS. 1 AND 2.

Fig. 1.—Sperm-head of the Murine type.

Fig. 2.—Sperm-head of the Microtine type.

Both figures are labelled to illustrate the nomenclature used in this study. Cytoplasm is indicated by a broken line.

A.R., anterior recess; C.A., apex of head-cap; D., dorsal side of sperm-head; D.E., dorsal eave; D.S., dorsal spike; D.P.R., dense posterior region of the nucleus; H.C., head-cap; I., insertion; I.N., insertion notch; L.C.L., lateral head-cap limit; M.P., middle-piece; N.A., nucleus apex; R., rear; R.D., rod; V., ventral side of the sperm-head; V.C.L., ventral head-cap limit.

Type 1. Primitive.—The sperm is small with a round unflattened head and a short middle-piece; e.g. *Hystrix* (30 $\mu$  long).

Type 2. Somewhat Specialized.—The sperm is bigger than the first type and has an oval flattened head, a head-cap of no great size, and a short thin middle-piece; e.g. *Dipus* (60 $\mu$  long).

Type 3. More Specialized.—The sperm-head is very flat

and its long axis is set at an angle to the tail. The head-cap is large and covers the front and sides of the nucleus. The middle-piece is comparatively short and thin; e.g. *Cavia* ( $110\mu$  long).

Type 4. Most Specialized.—The Muride type. The head is flat and shaped like a bill-hook, while the head-cap is smaller than in the previous type. The spiral filament on the middle-piece is particularly well developed; e.g. *Apodemus* ( $133\mu$  long).

I have added some estimated sizes to these descriptions of Retzius so that the types may be compared numerically. It will be shown later that two genera of the Muridae, at least, do not conform to this most specialized pattern. Retzius figures and describes the sperms of six Muridae, three of which (the Norwegian Lemming, *Lemmus lemmus*; the Brandmaus, *Apodemus agrestis*; and *Microtus arvalis*) are not now found in this country. The others, however, *Apodemus* (*Mus*) *sylvaticus*, *Mus musculus*, and *Rattus* (*Mus*) *norvegicus*, are still represented in Great Britain. His figures are enlarged camera lucida drawings, but from the point of view of a subsequent worker the lack of a scale is regrettable.

Other workers, notably Ballowitz (2), Duesberg (5), Mârza (9), and Regaud (14) have described the sperms of the rat or the mouse, but I have been unable to find descriptions of other species outside the work of Retzius.

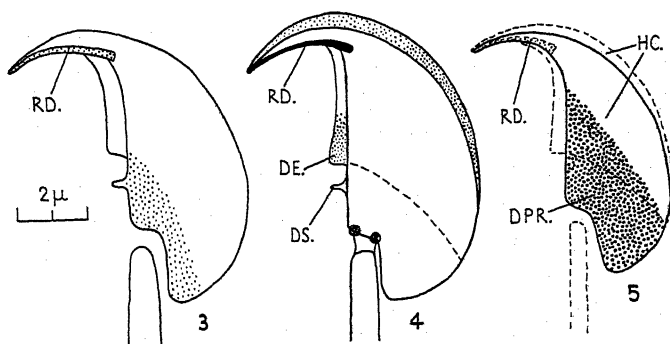
## VI. DESCRIPTIONS OF SPERMS.

### (i) Murinae.

#### *Mus musculus*.

I have examined the sperms of the 'wild' variety of the house mouse and those of some of the fancy and albino breeds, but can detect no significant differences of detail or size between them. These sperms are well known and I have little to add to existing accounts. Mârza (9), in a very interesting study, has investigated the histo-chemistry of the sperms of the mouse, the rat, and other mammals, using Feulgen's technique to determine the limits of the nucleus and head-cap. In the house

mouse, preparations by this method show (Text-fig. 5) that the nucleus extends almost to the tip of the head-cap. The light green counter-stain, however, does not stain the dorsal spike. Presumably this structure is destroyed by one of the rather rigorous processes of this technique, for it is well seen in fresh



TEXT-FIGS. 3-5.

Fig. 3.—*Mus musculus*, unstained, from saline mount.

Fig. 4.—*Mus musculus*, from osmium-haematoxylin-balsam preparation.

Fig. 5.—*Mus musculus*, from a Feulgen and light-green preparation; cytoplasm shown by broken line.

Lettering as in Text-figs. 1 and 2.

sperms and in those stained in basic fuchsin, gentian violet, and haematoxylin (Text-fig. 4).

One sperm taken at random from a standard mount was  $124\mu$  in length, approximately, this being made up of head  $8\mu$ , middle-piece  $21\mu$ , and tail  $95\mu$ . The mean total length of forty sperms was  $125.5 \pm 0.5\mu$ , S.D. being  $2.1\mu$ . Similarly the mean head-length of fifty was  $8.27 \pm 0.04\mu$ , S.D. being  $0.31\mu$ .

*Apodemus sylvaticus* and *Apodemus flavicollis*.

There seems to be little doubt that these two species are valid. The first, the long-tailed field mouse, is generally distributed throughout Great Britain, whereas the other, De Winton's field mouse, is less common, though not rare, being found in isolated localities in the south of England. My specimens came from Essex, and I have to thank Mr. C. Richardson for sending them

to me. De Winton's mouse used to be looked upon as a variety of *Apodemus sylvaticus*, but Hinton (3) and Miller (10) consider it to be a distinct species. The two mice are distinguished by their size and pelage and, although they occur together in some localities, they do not, according to Thompson (16), interbreed.

The sperms of the two species are almost identical in shape (Text-figs. 6 and 7). They differ in size however, for two animals, one of each species, killed within a few minutes of one another had sperms with the following mean head-lengths, *Apodemus sylvaticus*,  $9.83 \pm 0.04 \mu$ , *Apodemus flavicollis*,  $8.78 \pm 0.04 \mu$ . A similar pair averaged  $9.56 \pm 0.04 \mu$  and  $8.64 \pm 0.04 \mu$  respectively. This difference in head-length is paralleled by a difference in the total length of the sperms of the order of  $7 \mu$  (*Apodemus sylvaticus*  $132.8 \pm 0.5 \mu$ , *Apodemus flavicollis*  $125.4 \pm 0.5 \mu$ ). The total length in two sperms chosen at random from the two species was made up as follows: head  $9.75 \mu$  and  $8.75 \mu$ , middle-piece  $24 \mu$  and  $23 \mu$ , tail  $99.5 \mu$  and  $93.25 \mu$  respectively.

The sperms of the genus *Apodemus* are somewhat like those of *Mus* in having an overhanging dorsal eave, a dorsal spike, and a rod, but the anterior hook is much more pronounced.

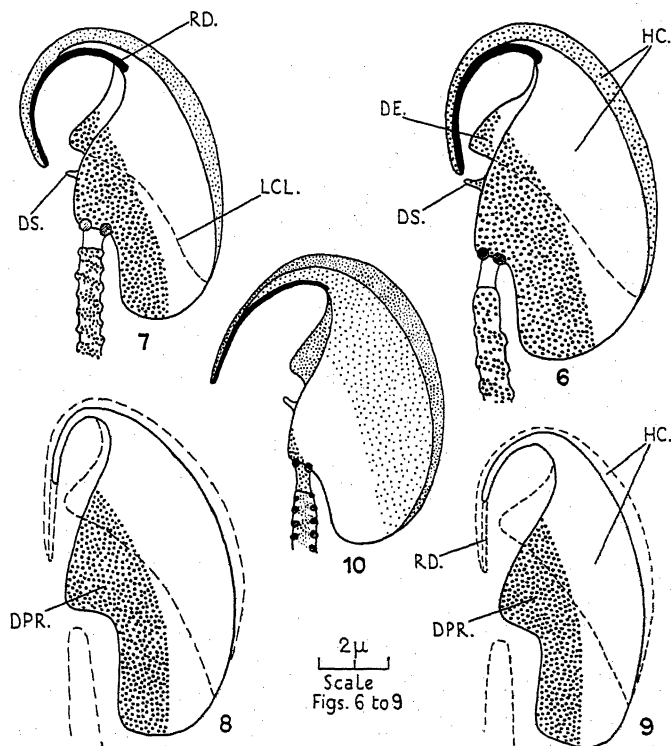
Retzius figures (Text-fig. 10) the sperm of *Apodemus agrestis*, the Brandmaus of central and eastern Europe. It is very like those of the two British species but, since it has not been measured, it is impossible to say if it is distinguishable from them.

#### *Rattus norvegicus* and *Rattus rattus*.

The two British rats, the common rat and the black rat, have similar sperms. Compared with those of the house mouse, the heads are drawn out along the antero-posterior axis and bent dorsally, about half-way along this line, to form the anterior hook. The anterior three-quarters of the nucleus is covered by the head-cap, which has a dorsal eave. The rod is strongly stained in most preparations, but there is no dorsal spike.

I have examined the sperms of the wild common or brown rat and those of the laboratory white rat, which is usually

regarded as a variety of the first. Their sperms certainly confirm this. I am particularly grateful to Mr. Colin Matheson who sent me living specimens of the black rat from Cardiff Docks,



TEXT-FIGS. 6-10.

Fig. 6.—*Apodemus sylvaticus*, from osmium-haematoxylin-balsam preparation.

Fig. 7.—*Apodemus flavicollis*, as in fig. 6.

Fig. 8.—*Apodemus sylvaticus*, from Feulgen preparation; cytoplasm indicated by broken line.

Fig. 9.—*Apodemus flavicollis*, as in fig. 8.

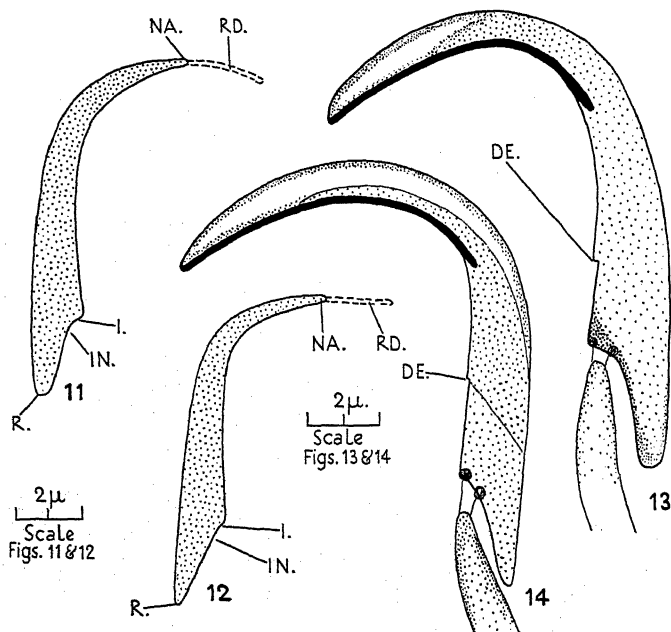
Fig. 10.—*Apodemus agrarius*, after Retzius; not to scale.

Lettering as in Text-figs. 1 and 2.

so that I was able to see the sperms of these three kinds of British rat.

The sperms of the two species are well differentiated both in total length, head-length, and in morphological detail.

The mean total length of sample sperms from each kind were found to be ( $n$  being the number measured):



TEXT-FIGS. 11-14.

Fig. 11.—*Rattus norvegicus*, camera lucida drawing from Feulgen preparation showing nucleus and rod only.

Fig. 12.—*Rattus rattus*, as in Text-fig. 11.

Fig. 13.—*Rattus norvegicus*, from an osmium-haematoxylin-potassium acetate preparation.

Fig. 14.—*Rattus rattus*, from a saline mount tinged with gentian violet.

Lettering as in Text-figs. 1 and 2.

*R. norvegicus* (white),  $188.7 \pm 0.5 \mu$  S.D.  $2.0 \mu$   $n = 14$ .

*R. norvegicus* (brown),  $190.0 \pm 0.5 \mu$  S.D.  $2.0 \mu$   $n = 11$ .

*R. rattus* (black),  $165.6 \pm 0.6 \mu$  S.D.  $1.8 \mu$   $n = 10$ .

The difference between the first two ( $1.3 \pm 0.7 \mu$ ) is not significant for the numbers measured, but a significant difference of

$23.1 \pm 0.8\mu$  exists between the first and last and a similar one between the second and last. These differences arise mainly from the length of the tail, which in *Rattus norvegicus* is  $110\mu$  long, approximately, while that of its congener is  $90\mu$ . The sperms of other specimens of these three kinds of rat had total lengths which fell well within the above limits, thus confirming the difference in total length between the sperms of the two species.

Total length, since it means, in most cases, measuring a curved sperm, is not an easy determination, and it is of little use for rapidly discriminating between two samples. The sperm-heads, however, of the two species of rat differ in a number of morphological features which enable them to be distinguished at sight. Examples from Feulgen and light-green preparations (Text-figs. 11 and 12) show these differences quite clearly. The chief are to be found in the rear of the nucleus. In *Rattus rattus* the insertion is comparatively small, the insertion-notch shallow, and the rear is less solid and rounded, while the dorsal eave, not well seen in Feulgen preparations, is smaller than in *Rattus norvegicus*. The posture of the hook varies slightly in the two species but, since this is difficult to estimate by eye, it cannot be used conveniently as a criterion of specific difference.

The head-lengths of random samples of fifty sperms from the two varieties of the common rat had the following means:

White	.	.	$11.72 \pm 0.04\mu$	S.D. $0.30\mu$ .
Brown	.	.	$12.05 \pm 0.05\mu$	S.D. $0.33\mu$ .

The difference between the two is  $0.33 \pm 0.06\mu$ ; its significance will be discussed below (p. 437). The head-lengths of a hundred and fifty sperms from the black rat had a mean of  $10.82 \pm 0.03\mu$ , S.D. being  $0.35\mu$ . The differences between the white and brown rats and the black rat are  $2.31 \pm 0.08\mu$  and  $2.44 \pm 0.08\mu$  respectively, and are of a different order from that between the two varieties.

It is therefore abundantly clear that the sperms of these two species of the genus *Rattus* have developed a recognizable difference of shape and size.



The sperms of *Rattus rattus* were used in a study designed to see if any measurable differences could be discovered between sperms taken from the caput of the epididymis and those from the cauda. The epididymis from a fecund animal was divided into three parts which were mounted separately by the standard and other techniques. The mean head-lengths of fifty sperms in each case were as follows:

Caput	.	.	$10.86 \pm 0.05 \mu$	S.D. $0.38 \mu$ .
Intermedia	.	.	$10.79 \pm 0.05 \mu$	S.D. $0.35 \mu$ .
Cauda	.	.	$10.82 \pm 0.05 \mu$	S.D. $0.38 \mu$ .

The differences between these means are not significant, and the same result was obtained for other similar preparations. No appreciable size change takes place, therefore, as the sperms travel down the epididymis of a fecund black rat.

#### *Micromys minutus*.

The sperms of the harvest mouse have not yet been described, possibly because the animals, although not uncommon, are difficult to procure. It is doubtful if they can be trapped, and all the specimens I obtained were caught by hand. They came from three sources, two in Essex and one in Sussex, and I am most grateful to Mrs. Hayward and to Messrs. Chalk and Butler for getting them for me.

The sperm of *Micromys* (Text-figs. 15-18) is unlike the usual Muride sperm in having no anterior hook, although it has a flattened and asymmetrical head. Both the head-cap and the nucleus have a longer border on one side than the other and the dense posterior region tends to be restricted to regions of the insertion and the rear. There are two centrosomes and a middle-piece spiral of thirteen or fourteen turns, but there is no rod and no dorsal spike.

The total length of a sperm taken at random from a standard preparation was  $68.75 \mu$ , made up of head  $5.75 \mu$ , middle-piece  $13.25 \mu$ , and tail  $44.75 \mu$ , the average total length being  $63.9 \pm 0.8 \mu$ . The mean head-length of a sample of fifty sperms was  $5.67 \pm 0.04 \mu$ , S.D. being  $0.29 \mu$ . Thus this sperm is smaller than any Muride sperm hitherto described. Its general shape

and size suggest that it may be a primitive type, and in this it is like the sperm of the musk rat (see p. 434).

(ii) *Microtinae*.

*Microtus hirtus*.

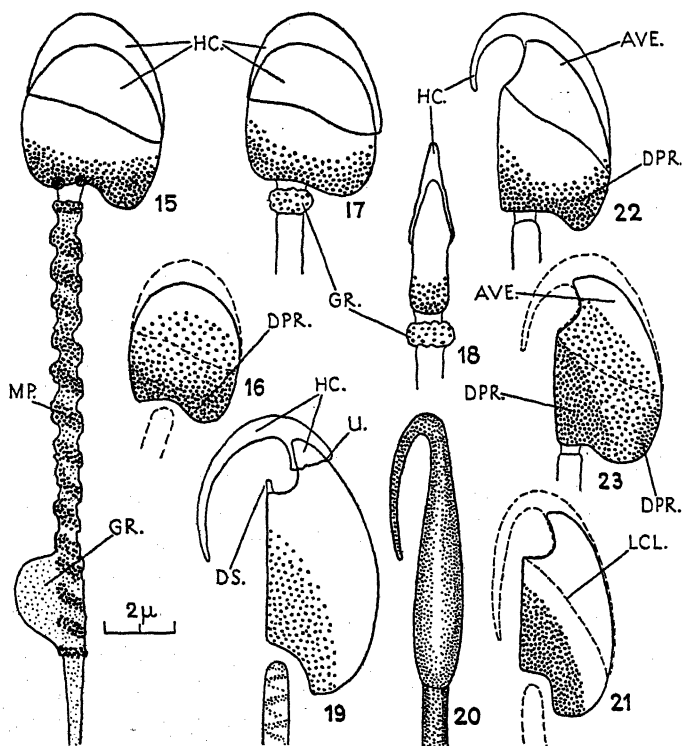
The sperms of the southern species of the short-tailed field mouse resemble those of the other *Microtines*, *Evotomys*, *Dicrostonyx*, and *Lemmus*. In these the nucleus-apex lies ventral to the main dorsal edge and thus an anterior recess is formed, whereas in *Mus*, *Rattus*, and *Apodemus* the anterior part of the nucleus lies dorsal to this line and forms the characteristic nucleus-hook. These two types are contrasted in Text-figs. 1 and 2.

The sperms of *Microtus hirtus* (Text-figs. 19, 20, and 21) have a straight dorsal edge which lies parallel to the normal axis of the tail. It is approximately  $4\mu$  in length and bears at its anterior end a forwardly directed dorsal spike, not seen in Feulgen preparations. The head-cap forms a long hook, which is made up, in contrast to that of the *Murinae*, almost entirely of extranuclear material. Occasional sperms are seen (Text-fig. 19) in which the head-cap is very much shrunk away from its normal position. Its lateral limit usually extends from the level of the dorsal spike, on one side, to a point opposite the insertion on the other (Text-fig. 21).

The total length of one sperm chosen at random was  $118\mu$ , made up of head  $7.5\mu$ , middle-piece  $30\mu$ , and tail  $80.5\mu$ . The mean total length was  $117.2 \pm 0.4\mu$ , S.D. being  $1.3\mu$ , while the mean head-length of fifty sperms was  $7.64 \pm 0.05\mu$ , S.D. being  $0.33\mu$ .

*Evotomys glareolus*.

The sperms of the bank vole are somewhat like those of *Microtus* (Text-fig. 22), but their total length,  $86.7 \pm 0.4\mu$ , S.D. being  $1.2\mu$ , is less and the head bears a much shorter hook. Less obvious points of difference are the slightly convex dorsal edge of the sperm-head of *Evotomys*, its smaller anterior recess and its shorter insertion-notch. There is no dorsal spike in *Evotomys*. In Feulgen preparations (Text-fig. 23) the dense posterior region appears as two densely staining patches, which are not joined together as they are in



TEXT-FIGS. 15-23.

Fig. 15.—*Micromys minutus*, from an osmium-basic fuchsin-potassium acetate preparation.

Fig. 16.—*Micromys minutus*, from Feulgen preparation; cytoplasm shown by broken line.

Fig. 17.—*Micromys minutus*, from a saline mount tinged with basic fuchsin. The ventral edge of the head-cap is displaced.

Fig. 18.—*Micromys minutus*, from the same tinted mount as Text-fig. 17. A sperm-head seen sideways.

Fig. 19.—*Microtus hirtus*, unstained sperm in 4 per cent. formaldehyde. The position of the lateral limit of the head-cap is unusual. Compare with the normal lateral limit in Text-fig. 21.

Fig. 20.—*Microtus hirtus*, unstained sperm in 4 per cent. formaldehyde, seen sideways.

Fig. 21.—*Microtus hirtus*, from Feulgen and light-green preparation; cytoplasm shown by broken line.

Fig. 22.—*Evotomys glareolus*, from osmium-basic fuchsin-balsam preparation.

Fig. 23.—*Evotomys glareolus*, from a Feulgen and light-green preparation; cytoplasm shown by broken line.

Lettering as in Text-figs. 1 and 2. A.V.E., thin anterior ventral edge of nucleus; G.R., Golgi remnant; U., unusual lateral limit of head-cap.

standard mounts. Another unusual feature is that the antero-ventral edge of the nucleus is very lightly stained by Schiff's reagent. This is presumed to be due to its thinness at this point.

A random sample of fifty sperms from a standard preparation of the bank vole had a mean head-length of  $6.85 \pm 0.02 \mu$ , S.D. being  $0.16 \mu$ , one typical sperm being  $85.25 \mu$  in length, made up of head  $6.75 \mu$ , middle-piece  $19.5 \mu$ , and tail  $59.0 \mu$ . In all these respects the sperm of *E. votomys* is smaller than that of *Microtus*.

#### *Dicrostonyx* sp.

I was able to get one male specimen of this New World lemming, but have only an incomplete series of preparations of its sperms. It was an old animal and there were few sperms in the epididymes. I found it impossible to counter-stain the head-cap after Feulgen (Text-fig. 24), but sperms from the testis (Text-fig. 25) show the sperm-head complete with head-cap. From these it can be seen that this lemming has sperms which resemble those of *Microtus*, but the anterior recess is shallower and the posterior breadth of the nucleus is greater. The much longer insertion-notch ( $1.5 \mu$  approx.) distinguishes these sperms, in turn, from those of *E. votomys*, in which it is much shorter ( $0.5 \mu$  approx.).

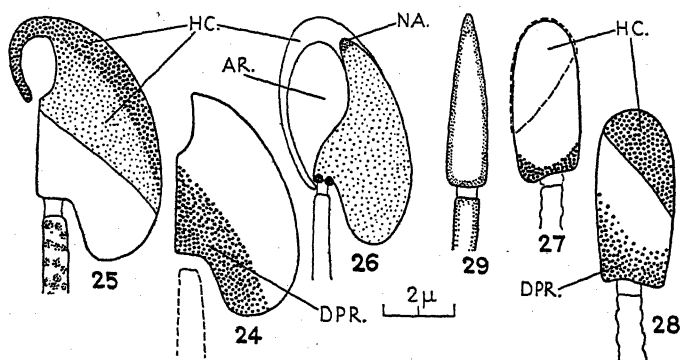
#### *Lemmus lemmus*.

I am including a figure of the sperm of the Old World lemming (Text-fig. 26) from Retzius. This is of the Microtine type, but the dorsal edge of the nucleus is unusually short and the anterior recess is correspondingly large. The hook is the longest yet seen in this group. The sperms of this lemming and the previous one do not suggest any close relationship between the two genera.

#### *Ondatra zibethica*.

Although the musk rat is a member of the sub-family Microtinae its sperms are atypical and unlike those of *Microtus*. The head is flattened but unhooked, and the head-cap is a thin sheath investing the anterior part of the nucleus and not projecting beyond it (Text-figs. 28 and 29). As in the harvest mouse, this simplicity is conjoined with some features which

are shared by the more typical sperms of the Muridae. These are the flattened head and the asymmetry of the head-cap, nucleus, and dense posterior region, so that this sperm, like that of *Micromys*, might be interpreted as one which had lost or had never had an anterior hook. Both sperms are similar



TEXT-FIGS. 24-29.

Fig. 24.—*Dicrostonyx* sp., sperm-nucleus from a Feulgen preparation.

Fig. 25.—*Dicrostonyx* sp., sperm from section of testis—Flemming-haematoxylin-euperal vert.

Fig. 26.—*Lemmus lemmus*, after Retzius; not to scale.

Fig. 27.—*Ondatra zibethica*, from a Feulgen and light-green preparation; cytoplasm indicated by broken line.

Fig. 28.—*Ondatra zibethica*, from osmium-haematoxylin-balsam preparation.

Fig. 29.—*Ondatra zibethica*, from same preparation as Text-fig. 28; sperm seen from edge.

Lettering as in Text-figs. 1 and 2.

to that of *Dipus*, which it will be recalled Retzius described as being typical of the second or 'somewhat specialized' of the classes into which he divided the sperms of the Muridae.

A sperm of *Ondatra*, chosen at random from a standard preparation, possessed a head  $5.25\mu$  long, a middle-piece  $16.25\mu$  long, and a tail  $48\mu$  long, the total length being  $69.5\mu$ . The mean total length was found to be  $67.7 \pm 0.4\mu$  and the mean head-length  $5.39 \pm 0.04\mu$ , S.D. being  $0.31\mu$ . The coefficient of variation of head-length was 5.7, but the standard deviation is quite normal for the group.

It will be noticed that in size, as well as in shape, the sperms of the musk rat agree with those of *Micromys* and *Dipus*.

#### General observations.

From the foregoing it will be seen that the sperms of the Muridae have flattened heads, which are always asymmetrical but not, as Retzius believed, always hooked.

The Muride head-cap, which is also asymmetrical, tends, when stained and fixed, to be inconstant in shape and position. In those species in which the hook is unsupported, either by a part of the nucleus or by the rod, it may show varying degrees of curvature; while in some preparations the thin lateral portions of the head-cap show signs of having been detached from the rest and shrinking forward. It is apparent that, with the possible exception of the dorsal spike, seen only in *Mus*, *Apodemus*, and *Microtus*, the head-cap is the most delicate organ of the sperm. This is confirmed by Baker (1) who found that, in the cavy, the head-cap is more easily damaged and distorted by reagents than any other part.

Two centrosomes, placed at the insertion of the neck, are a constant feature of the group.

To avoid complication, the lengths of the neck and end-piece have not been given in the descriptions of the sperms of the species. The neck, in these, varies in length from  $0.5\mu$  to  $1.5\mu$ , but this could safely be omitted from a comparative survey, since it is short and its length is included in that of the head. Similarly the length of the end-piece ( $1\mu$ – $5\mu$ ) is included in that of the tail.

#### VII. VARIATION IN SPERMS.

Sperms taken from the same animal, like any other cells from a similar source, vary slightly in morphological detail, but these minor differences are never sufficient to obscure the structure which is associated with each genus. Abnormal and giant sperms occur from time to time but, since they are surrounded by so many normal ones, the abnormality is obvious and there is never any difficulty in identification.

Sperms, too, as has been shown in the preceding section, vary

in size, the head-lengths, in particular, of sperms from standard preparations being found distributed approximately normally about the mean for each individual mouse and rat. It is still, however, an open question whether this distribution is unimodal or bimodal, and the problem is confused by some workers measuring the nucleus length while others measure the whole head of the sperm. Zeleny and Faust (18) measured the nucleus-length of large numbers of sperms from several insects and mammals and their work affords striking evidence of bimodality; while Parkes (13) measured the complete head-lengths of smaller numbers of sperms of some mammals and showed bimodality in them. Williams, Savage, and Fowler (17), on the other hand, examining the sperms from a large number of bulls, obtained no examples of bimodality of complete head-length, all distributions being unimodal. I am in no position to decide between these two beliefs, but in the relatively small numbers of sperms whose head-lengths sufficed for my work, I found little evidence for bimodality. In three cases out of about forty I obtained distributions of this type—1, 4, 2, 8, 15, 18, 5, 5, 1, 1. The small peak at 4, however, was not taken to be a mode but to be due to uneven sampling. In the remaining cases the distributions approximated to the normal.

These variations in apparent head-length are due, in the main, to that inherent variability which is found in any population, but some differences in size must also be due to factors of technique and observation. Errors of this kind, which could be foreseen, were guarded against and every effort was made to ensure that conditions of work were as uniform as possible and that the sperms measured were taken at random.

When sperms from different males of the same species were measured it was found that the resulting mean head-lengths differed considerably. This will be seen from the data for four specimens of the house mouse given below. The mice were dealers' specimens which from their coat-colour seemed to be mixed genetically.

Mouse	A	B	C	D
Mean head-length in $\mu$	$8.00 \pm 0.04$	$8.27 \pm 0.04$	$7.84 \pm 0.04$	$7.98 \pm 0.04$
Coefficient of variation	4.3	3.4	3.5	3.7

These results might be interpreted as being due to the random sampling of a homogeneous population of sperms. The difference of  $0.43 \pm 0.06\mu$ , however, between Mouse B and Mouse C makes this explanation extremely improbable, for in this case the ratio of the mean to the standard error (subsequently referred to as  $t$ ) is 7.5, which is well in excess of the 1.9 limit of significance for  $t$  when the number measured is, as in this case, a hundred.

The most acceptable explanation of this wider variation is that the mean head-length of its sperms is a character associated with an individual mouse comparable with, for example, other characters such as the weight of its body or the length of its tail. These last, it is well known, vary normally throughout a population, and it is reasonable to expect that the mean head-length of the sperm will likewise vary.

Assuming this to be true for the small group of mice quoted above, it can be shown that what will be called the primary means are distributed about a secondary mean of  $8.02 \pm 0.09\mu$  with a standard deviation of  $1.8\mu$  and a coefficient of variation of 2.2. Making all allowance for small numbers this indicates that the secondary variation in the length of sperm-heads is smaller than the primary.

Taking the usual limits of significance as a probability of 0.05, the primary means should rarely exceed a value of twice the standard deviation on each side of the secondary mean. That is to say that, in this case, the primary mean head-length will rarely be found outside the values  $7.66\mu$  and  $8.38\mu$ . The difference between these extremes is  $0.72 \pm 0.06\mu$ , for which  $t$  is 12, a value which will be found useful below (p. 488).

The following differences between the primary means for different individuals of the same species were also obtained:

<i>Species</i>	<i>Difference in <math>\mu</math></i>	<i>Value of <math>t</math></i>
<i>Mus musculus</i> . . .	$0.43 \pm 0.06$	7
<i>Apodemus sylvaticus</i>	$0.27 \pm 0.06$	5
<i>Apodemus flavicollis</i>	$0.14 \pm 0.05$	3
<i>Rattus rattus</i> . . .	$0.38 \pm 0.06$	6



In these pairs the primary means are separated by significant differences which, it is believed, are the result of a secondary variation. It should be noticed that in no case does the value of  $t$  approach 12.

A variation was found in the mean total lengths of the sperms of the same four mice, but in no case was the difference between them large enough to suggest that the total lengths of the sperms of *Mus musculus* were not distributed homogeneously throughout the species.

#### VIII. SPECIFIC AND GENERIC DIFFERENCES.

Two pairs of congeneric species were examined—the two British rats and the two British long-tailed field mice. In the first two the sperms of *Rattus norvegicus* could be distinguished at sight from those of *Rattus rattus*, and there were also significant differences in head-length of  $0.90 \pm 0.05\mu$  and  $1.23 \pm 0.06\mu$ . These give values for  $t$  of 18 and 20 respectively. Two parallel sets of congeners from the genus *Apodemus* showed the following differences in head-length, the first  $1.05 \pm 0.06\mu$ ,  $t$  being 18, and the second  $0.92 \pm 0.05\mu$ ,  $t$  being again 18.

In these cases the values of  $t$  are larger than those given in the preceding section and they suggest that the calculated limiting value of 12 for  $t$  has a general application to sperms other than those of *Mus*. It is not believed that here is an infallible quantitative method of discriminating between varieties and species generally but that in these two genera of the Muridae it certainly holds good. Subsequent work may modify this value of  $t$ , but for these Muridae it must lie between 7 and 18.

The basis of these differences is ultimately genetic, and a passage from Haldane (8, p. 82) is apposite enough to be quoted. He sums up the genetic aspect of group differences in the animal and plant kingdoms as follows: 'Interspecific differences are of the same nature as inter-varietal. But the latter are generally due to few genes with relatively large effects, and rarely to differences involving whole chromosomes or part of them. The reverse is true of differences between species. The number of

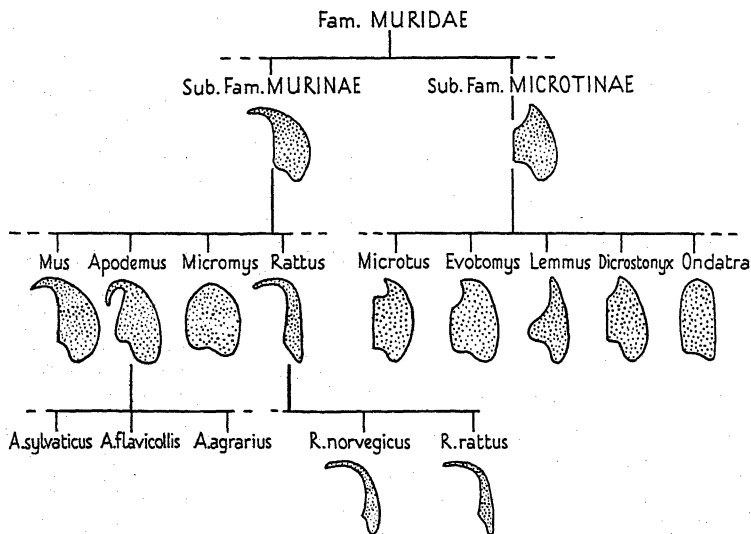
genes involved is often great and cytologically observable differences common.' The latter part of this is illustrated by Cross (4) who finds differences not only in chromosome number between several species of North American rodents, but also in the morphology of analogous chromosomes. These differences, he believes, are due to fragmentation, inversion, and deletion with translocation but not to duplication. He also shows that the chromosome morphology of related sub-species may be quite distinctive.

It might be expected that such differences in the number and morphology of the chromosomes would result in observable differences in the size and shape of the nucleus of the sperm, but this need not be so, for if rearrangements only of the chromatin and not duplications are responsible for these differences no change in chromatin volume will be seen from species to species. Cross holds that this is true and states that he has calculated the volume of chromatin for the various species and finds it to be uniform for the Rodentia. He does not give his data for these calculations. The measurements on which they were based must have been very intricate and liable to error. Quite apart from this I find it most difficult to believe that the nuclei of all species I have seen have the same volume. The obvious difference of nuclear size between, for instance, the sperm of *Ondatra* and that of *Microtus* may have another explanation, for the chromatin volumes of the spermatogonia of each of the genera may be equal, but in spermatogenesis condensations or attenuations of nuclear matter may be responsible for these final differences.

Generic differences do not adapt themselves to genetic analysis, but they are almost certainly of the same nature as specific ones. In this study of sperms generic differences were so obvious microscopically that it was unnecessary and also impracticable to submit them to measurement. The sperms of *Rattus*, *Mus*, and *Apodemus*, for instance, are immediately distinguishable and characteristic for those genera, although older systematists included the three animals in the original genus *Mus*.

The problem of the meaning and function of these differences

in sperms is interesting. Like other minute structures entering into the anatomy of animals, such as hairs, scales, and blood-corpuscles, they are mainly evidences of the fundamental specificity of those animals. The distinguishing characters I have used in this study—the shape of the nucleus and head-cap, the head-length, and the total length must all be determined by specific factors acting during spermatogenesis. They are probably non-adaptive and due to the secondary effects of some



TEXT-FIG. 30.

Diagram summarizing the morphology of the nucleus in the sperms of the Muridae.

elements in the gene-complex which have major effects on the soma. There is no evidence to suggest that even the typical hooked sperm or the rod has an adaptive function or confers any advantage on the animal which bears it. There is no need, therefore, for the sperms of different animals to differ visibly, but so complete is the specificity of animals that in most cases, together with other anatomical features, they probably do so.

## IX. SUB-FAMILY DIFFERENCES.

It has already been shown (p. 423) that within the family Muridae, a Microtine and a Murine type of sperm-head can be distinguished. This distinction, however, breaks down when the sperms of *Micromys* and *Ondatra* are considered; for both these animals, one a Murine and one a Microtine, have simple unhooked sperms. The presence of this relatively simple sperm-head in both sub-families suggests that the characteristic hooked sperm has been evolved separately in each of these groups and that the primitive Muride sperm was not hooked. Further descriptive work on the sperms of the Muridae, especially on the more primitive Cricetinae, is necessary before this can be confirmed. So far the sperms of only a few Muridae have been described and there remain literally hundreds of species which have not yet been examined.

## X. SUMMARY.

1. The spermatozoa of the following Muridae, which had not previously been described, have been studied in detail, measured, and figured:

*Apodemus flavicollis* (De Winton's field mouse).

*Micromys minutus* (harvest mouse).

*Rattus rattus* (black rat).

*Microtus hirtus* (short-tailed field mouse).

*Evotomys glareolus* (bank vole).

*Dicrostonyx* sp. (Canadian lemming).

*Ondatra zibethica* (musk rat).

In addition the sperms of the following, described and figured by Retzius, have been measured:

*Mus musculus* (house mouse).

*Apodemus sylvaticus* (long-tailed field mouse).

*Rattus norvegicus* (common rat).

2. Among these, two distinct types of sperm can be distinguished, one with a hooked nucleus associated with the sub-family Murinae and the other with a recessed nucleus associated with the sub-family Microtinae. In both of these, however, the head as a whole is hooked.

3. The sperms of all the genera hitherto described and those described in this paper fall into these two type-groups, with the exception of those of the harvest mouse and the musk rat, the heads of which are simpler and unhooked.

4. It is believed that the simpler type of sperm is the more primitive, since it is similar to those found in more primitive groups of the Rodentia; and that the hooked head has been evolved independently in the two sub-families.

5. Each of the genera examined has a distinctive sperm; but specific differences are not so obvious, the sperms of the two British rats, *Rattus norvegicus* and *Rattus rattus*, differing in minor features and in head-length, while those of the two species of *Apodemus* differ in head-length alone.

6. Within each species, however, differences in mean head-length occur between individuals, and these are sometimes significant. It appears that the mean head-length of its sperms is a characteristic of each male rat or mouse comparable to other characteristics such as the length of its tail. It is presumed that this cytological characteristic, like the grosser ones, varies normally throughout the population.

7. It is shown that a quantitative criterion exists for deciding, from the head-length of their sperms alone, whether two individuals of either the genus *Apodemus* or the genus *Rattus* belong to the same species or represent distinct species. Since these were the only two genera in which congeneric species were examined, it is not known whether this criterion may have a more general application.

8. In each of these groups of the Muridae the morphology of the sperm has evolved with the evolution of the animals which bear it. Thus it is possible to recognize any of these species from their sperms alone, and to decide in the same way the genus and (with two exceptions—*Micromys* and *Ondatra*) the sub-family to which they belong.

9. Outlines of the nuclei of all the known sperms of the Muridae, taken wherever possible from Feulgen preparations, are embodied in a diagram (Text-fig. 30) which places them in their natural groups and at the same time summarizes the

morphological side of this study. Other diagrams (Text-figs. 1 and 2) illustrate a nomenclature for the more specialized features of the Muride sperm.

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# The Development of the Amphibian Kidney. Part III. The Post-Metamorphic Development of the Kidney, and the Development of the Vasa Efferentia and Seminal Vesicles in *Rana Temporaria*.

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With Plates 20 to 23 and 3 Text-figures.

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## INTRODUCTION.

THIS research forms Part III of the writer's investigations into the development of the amphibian kidney, the two previous parts (Gray, 1930, 1932) having dealt with the development of the kidney of *Rana temporaria* and *Molge vulgaris* up to a stage slightly beyond metamorphosis. The post-metamorphic development of the kidney units themselves shows little of interest, but the method of their attachment, especially



in the post-metamorphic posterior region, is quite different from that of the earlier generations. Our knowledge of the development of the genital connexion, moreover, is very confused.

It is not the writer's intention, either here or in the future, to redescribe the development of the oviduct, since he is convinced, from his own observations, that the accounts of Hall (1904) and MacBride (1892) are substantially accurate. It is in the formation of the vasa efferentia, and in the passage of the sperm through the kidney, that the main gaps in our knowledge occur. The development of the seminal vesicle appears never to have been worked out.

#### A. MATERIAL AND METHODS.

The material of this research is substantially that used by the writer in his previous work on the frog (Gray, 1930) and will be found fully described in that paper. The post-metamorphic material has been collected from time to time, mostly in Norfolk. The young frogs were fixed in Bouin and stored in 70 per cent. alcohol before the urinogenital organs were dissected out. These have therefore been fixed in the shape they had during life, and fig. 5, Pl. 20, and fig. 30, Pl. 23, for example, are revealing to those who picture the kidney as the smoothly oval structure which appears in dissections.

Sections attached to the slide by the ordinary albumen methods were found not to retain the blood corpuscles, and therefore sections, such as that shown in fig. 1, Pl. 20, likely to contain much blood were dipped in 0.1 per cent. celloidin in ether after de-waxing in xylol and passing through absolute alcohol. The ether was allowed to evaporate until diffraction colours appeared, when the slide was dropped into 50 per cent. alcohol.

The reconstructions shown in figs. 8 and 9, Pl. 21, were prepared by the special reconstruction technique worked out by the writer for his work on Triton (Gray, 1932).

#### B. POST-METAMORPHIC DEVELOPMENT OF THE KIDNEY.

##### (i) Attachment of units to archinephric duct.

No previous worker appears to have studied the post-metamorphic development of the kidney, and, in my previous work

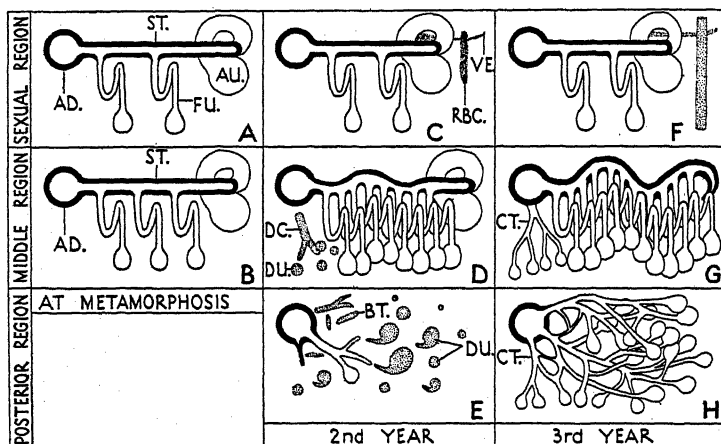
(Gray, 1930), I was handicapped by a lack of material taken between metamorphosis and sexual maturity in the fourth year. The story which I then put forward is accurate so far as it goes, but the three years following metamorphosis show great changes in the posterior (non-sexual) portion of the kidney, more especially with regard to the method of attachment of the units to the archinephric duct.

The position in *Rana* is particularly complicated and differs from *Triton* in that there is no clearly defined sexual region, for the anterior portion of the male kidney has some excretory function. The excretory and non-excretory regions grade into each other. Each has its own special system for the connexion of units to the archinephric duct. In the anterior, sexual region the 'straight tubules' described in my previous research form both the direct connexion between the vasa efferentia and the archinephric duct and also serve for the attachment of such excretory units as occur in this area. These straight tubules also occur in the mid-region of the kidney, posteriorly to the vasa efferentia, and there serve solely for the attachment of excretory units. They are not, however, sufficient in number to provide for the attachment of every unit, and large numbers of secondary, arborizing, collecting-trunks arise from the blastema which immediately surrounds the archinephric duct. During the course of the second and third years after metamorphosis the 'kidney' increases greatly in length, but this increase is due solely to the addition of units to the posterior region. No further transverse straight tubules are formed, but these new units are taken up by numbers of small collecting-trunks from the archinephric duct.

The fact that the kidney increases in length by additions to its posterior region is well shown by an examination of the relative position of the testis. Fig. 16, Pl. 22, shows the appearance of the urinogenital system of a 53-mm. *Rana temporaria*, taken and fixed in Bouin in July: that is, one which would have reached sexual maturity in about nine months' time. Even at this late stage the testis appears to lie relatively farther towards the posterior end of the kidney than in the adult. The posterior region of the kidney, moreover, is clearly differ-

entiated in the photograph both by its shape and by the greater quantity of blood which it contains.

The posterior augmentation of the kidney, and the variation in the types of attachment of the units must be clearly grasped before any interpretation of sections can be given,



TEXT-FIG. 1.

Diagram to show the methods of attachments of units to the archinephric duct between metamorphosis and sexual maturity. AD., archinephric duct; AU., abortive unit; CT., collecting trunks; BT., blind tubule; DC., developing collecting-trunk; DU., developing unit; FU., functional unit; RBC., rudiment of Bidder's canal; VE., vas efferens.

and the following explanation of Text-fig. 1 is therefore given before the description of the sections upon which it is based.

A and B show the condition throughout the entire kidney at metamorphosis. This is the condition which was described in my last paper, but may be recapitulated here. As the archinephric duct (AD.) has passed from the inner to the outer margin of the kidney it has left, lying transversely across the dorsal surface of the kidney the straight tubule (ST.) ending in an abortive malpighian unit (AU.). Functional units (FU.) have become attached to this tubule in both regions. For a fuller description of this process see Gray (1930, Text-figs. 6 and 7). What is here referred to as the middle region (Text-fig. 1, B) is

actually the hinder end of the kidney at metamorphosis, the true posterior region (Text-fig. 1, E and H) being added during the succeeding years.

In the sexual region (Text-fig. 1, C and F) the elaboration of the sexual connexion is the only change; this is described more fully in a later part of this paper. No further functional units are added, and the straight tubule maintains its primitive appearance.

In the mid-region, however (Text-fig. 1, D and G), there is a considerable increase in the number of functional units (FU.) which become connected to the straight tubule (ST.). This last loses its primitive straightness and becomes pulled out from one side to another as more and more tubules become attached. At the same time its histological appearance becomes less sharply differentiated from the excretory tubules which surround it. Thus there arises a condition when no further room can be found for such attachments, and a number of collecting-trunks (DC.) arise. These are produced in situ from the blastema which always surrounds the inner angle of the kidney. Units (DU.) also arise in this area, in the manner already described (*loc. cit.*), and become attached to the collecting-trunks. The whole course of the development of these units and of the collecting-trunks themselves is highly irregular. Thus in the middle region of a third-year kidney (Text-fig. 1, G) there are some units regularly attached to the straight tubules and others which communicate with the archinephric duct through an irregular system of branched, and even sometimes anastomosing, collecting-trunks. The abortive unit which terminated the original straight tubule finally degenerates, and it becomes very difficult to decide, even by reconstructional methods, what was a straight tubule and what is a secondarily developed collecting-trunk.

In the posterior region, which becomes differentiated during the beginning of the second year, there are no straight tubules. There are, however, a considerable number of functional units which are connected solely to the collecting-trunk network. These are shown developing in Text-fig. 1, E, and are diagrammatically represented at the conclusion of their development

in Text-fig. 1, H. As blindly ending tubules (*st.*, Text-fig. 1, E) are occasionally found scattered among the developing units and collecting-trunks in the posterior region, it is probable that such anastomoses as there are have been produced by the attachment of these blind tubules rather than by fusion between the trunks themselves. Moreover, it may be observed that there is no spare blastema surrounding the archinephric duct in the adult, and it seems probable that such blastema as remains, after the formation of collecting-trunks and units has ceased, may be used up in the formation of numerous little accessory collecting-trunks. That such an anastomosing mass exists is shown by the work of Stewart (1927), who records that 'under conditions of injection and dissection of the ramifications of the collecting duct tree, it was not possible to make out in many instances the junction of the collecting duct and its distal convolutions. It is probable that there are many more than seven orders in the collecting duct system'. His figures show clearly that he was working upon a collecting-duct of the mid or posterior region.

The existence of the straight tubule and its connexions is very clearly seen in sections of the sexual region. Fig. 7, Pl. 20, represents a section across the extreme anterior end of the kidney of a second-year frog. The straight tubule (*st.*) ends in the abortive unit (*au.*). This most anterior of straight tubules is without excretory connexions. Fig. 6 on the same plate is from about 3 mm. farther back in the same series; the straight tubule (*st.*) is still equally distinct but shows quite clearly the attachments (*ae.*) of two functional units. At this period there is no trace of collecting-trunks.

Collecting-trunks are best seen in sections of a third-year kidney. Three sections from the same series of a 53-mm. frog are shown on Plate 20. The most anterior of these, fig. 5, Pl. 20, is from the middle region. The straight tubule (*st.*) is not nearly so sharply differentiated as it was in the two-year frog, but can still be distinguished from the surrounding tissues.

The attachments (*ae.*) to the functional units are best seen in the enlarged central portion of this figure reproduced as fig. 17, Pl. 22. By this time, however, collecting-trunks have

appeared and show as small ducts (*ct.*, fig. 5, Pl. 20) with deeply staining nuclei. Fig. 1, Pl. 20, is taken towards the hinder end of the kidney in the region where the archinephric duct (*ad.*) is just passing away from the main mass. The last collecting-trunk to be directly connected is shown leaving the archinephric duct and is seen to be surrounded by minor collecting-trunks (*ct.*). Several developing glomeruli (*dm.*) are cut in this section. The last section (fig. 2, Pl. 20) is taken through the true posterior kidney, well behind the point of separation of the archinephric duct. Even at this late age (three years) the tissues still consist largely of blastema in which many developing units appear. The collecting-trunks (*ct.*) which run backwards from the more anterior point of attachment can again be clearly differentiated by their histological structure.

The gradual loss of distinctness which is noticeable in the straight tubule is well illustrated by fig. 3, Pl. 20. This is through the middle region of a 42-mm. (second-year) frog. The straight tubule, which here serves solely for the attachment of excretory units, is beginning to coil in the manner indicated in Text-fig. 1, so that it appears cut in several places. It is more readily distinguishable from the surrounding excretory tissues than will be the case a year later (fig. 5, Pl. 20), but is markedly less obvious than it was a year before (figs. 6 and 7, Pl. 20).

To sum up, then, the methods of unit attachment shown in the post-metamorphic kidney, there are, passing from anterior to posterior:

- (1) One, or at the most two, anterior straight tubules devoted to the carrying of sperm.
- (2) A further series of three or four straight tubules which carry both sperm and excretory products.
- (3) About six 'straight tubules', later becoming bent, which carry only excretory products.
- (4) A number of posterior collecting-trunks which are produced irregularly and anastomose among themselves.

(ii) Production of Accessory Peritoneal Funnels.

It was noted in Part I of this investigation (this Journal, vol. 73, pp. 533-7) that there are two methods for the production

of peritoneal funnels. The first, the only one figured by previous writers, is by separation from a primary unit; the second is through the action of special funnel-forming tubules which follow the course of the surface veins along whose coelomic walls the funnels develop.

During the second and third years after metamorphosis even these special tubules appear insufficient to provide the very large number of peritoneal funnels which are apparently necessary to the animal. As noted (*loc. cit.*) the funnels are grouped mostly in that region of the kidney which lies against the testis. This region consists, even in the third year, largely of blastema. This is well seen in fig. 5, Pl. 20, where an accessory peritoneal funnel (*pf.*) lies in the middle of this darkly stained blastema mass.

These accessory funnels are formed *in situ* from the blastema, first appearing as a cylindrical aggregation of cells which soon becomes conical, with the base of the cone against the peritoneal wall. A lumen then appears and the inner walls of the hollow cone acquire cilia at about the same time as the inner end makes contact with a renal venule. These accessory peritoneal funnels are all much longer than those produced by the first two methods described and occasionally acquire a bend reminiscent of an excretory unit. There is no doubt that they represent complete units which, from their position, are out of the sphere of influence of a straight tubule and therefore do not acquire excretory connexions, and it is not difficult to imagine that this lack has led to the suppression of the malpighian capsule.

One of the most remarkable features of these long funnels is the close association of the long tail with the renal arterioles. In fig. 10, Pl. 22, for example, one of these funnels (*pf.*) is seen lying close to the venule into which it will ultimately open. The renal arteriole, lying to the right of the venule, was noticed to be of unusual size and, on being followed back to its source, was found to be directly connected to the renal artery and to throw out no branches before reaching the neighbourhood of the peritoneal funnel. After lying for a short distance in close association with the tail of the funnel, the arteriole loops back

into the main mass of the kidney where it splits, in the ordinary manner, into numerous branches to the glomeruli of secondary malpighian capsules, and to the excretory portions of the secondary tubules.

These accessory funnels increase their number by direct splitting from the end of the tail to the funnel, as is seen in fig. 15, Pl. 22. It will be noticed also that the renal arterioles (*r.at.*) are similarly doubled, one branch being associated with each tail of the funnel. Only one of these tails is cut, and is seen lying just above the larger artery; the other runs directly away from, and in a plane at right angles to, the observer.

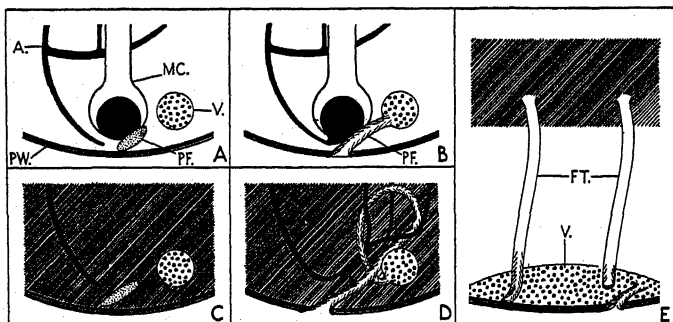
It is fairly universally accepted that the function of the glomerulus is confined to the adjustment of the water content of the blood and that the excretion of nitrogenous waste takes place through the renal tubules. It seems, therefore, very probable that the tails of these accessory funnels may have an excretory function, the waste products being passed to the arterioles. If this is not so, there is no apparent reason for the existence either of the tail, or of the special arteriole: if this postulate be accepted, then the production, function, and correlation of the whole series of peritoneal funnels becomes clearer.

In Text-fig. 2 are shown two stages in the production of a peritoneal funnel by each of the methods described. In A and B the funnel, closely associated with, but not opening into, the malpighian unit, directly connects the coelomic cavity with a venule. The artery supplies both the water-adjusting glomerulus and the waste-excreting tubule. This method is found only in young tadpoles and is a relic, of phylogenetic interest, of the urodele type. In E, which begins a few weeks before, and continues for a few months after, the tadpole leaves the water, special tubules connect the dorsal blastema mass to the peritoneum. In C and D, which begins during the first and continues during the second and third years spent by the frog on land, the blastema has shifted to the peritoneal surface and the tubules have acquired a tail with an arterial supply.

These observed facts of structure can be exactly correlated with the observed habits. As a tadpole the larva is not subject



to loss of water, and the return of peritoneal fluid to the veins is of small importance. As soon as it leaves the water, the circulation through the cutaneous vein must lead to a considerable increase in direct water-loss from the blood which must be directly replaced by the return of peritoneal fluid; large numbers of funnels are therefore produced at this period in the quickest possible manner. Such an arrangement, however, results in the mixing with the blood of large quantities of waste products



TEXT-FIG. 2.

Diagram to show the various methods by which peritoneal funnels are produced. The hatching represents undifferentiated blastema. A and B: from early units. C and D: accessory funnels from surface blastema. E: from funnel-forming tubules. A., artery; MC., malpighian capsule; FT., funnel-forming tubule; PF., peritoneal funnel; PW., peritoneal wall; V., vein.

picked up with the peritoneal fluid. Later funnels, therefore, are furnished with a short length of tubule with the aid of which the arteries can filter out the impurities from the peritoneal fluid and pass this to the excretory tubules proper. If the arteries had no special function to fulfil in this region they would scarcely be expected to run directly to the region of the funnels before passing to the kidney tubules.

### C. DEVELOPMENT OF THE VASA EFFERENTIA.

#### (i) Historical Summary.

The formation of the vasa efferentia and the course of the sperm through the kidney have been the subject of so much

controversy that a historical review of the subject appears to be justified.

The classic account of the male urinogenital organs of Amphibia is that of Bidder (1846). He first recorded that the sperm traverses the kidney and noted the sperm-duct, lying along the medial edge of the kidney, which to-day bears his name. Many papers on the amphibian urinogenital system appeared during the next thirty years, but none contributed anything to the problem of the genital connexion except a statement by Spengel (1876) that the ordinary excretory tubules have no connexion with the testis. It was left to Nussbaum (1880) to start a controversy which lasted twenty years.

This author, who appears to have been the first to study the development of the vasa efferentia, stated categorically that they were outgrowths from the wall of the malpighian capsule. These outgrowths grew through the mesorchium and became secondarily connected to an independently derived testicular network. The ducts from the kidney were apparent in a two-legged tadpole, but did not become attached to the testicular network till a few months after metamorphosis. Six years later the same author (Nussbaum, 1886) confirmed Spengel's observation, which had been made on *Rana esculenta* while investigating *Rana platyrhinus* [= *Rana fusca*]. In the same year Hoffman (1886) entered the field with the story that every mesonephric unit sent out a connexion to the genital strand while this latter was still in the undifferentiated condition, and that the posterior of these kidney-testis connexions degenerated after metamorphosis. Unfortunately, he also stated that the peritoneal funnels never open into veins (which can be disproved by the examination of almost any section—cf. Gray 1930, Pl. 28, fig. 7), so that his description was generally discounted on the score of faulty observation. It appears probable, nevertheless, that the condition he described is the primitive one in Amphibia, for it is very similar to that described by Semon (1890) for *Ichthyophis*. In this animal an epithelial strand runs out from the wall of each mesonephric duct, 'der sich in zwei Arme gabelt; der eine tritt zur Nebenniere, der andere zur Keimdrüse. Beide sind Derivate der ursprünglichen

Verbindung zwischen Nephrotom und Seitenplatten und zwar des inneren Theils dieser Verbindung.' These later degenerate in those segments in which sex-cells are not produced.

In 1897 Frankl investigated the sperm-passage by injections made through the archinephric duct. He stated in contradiction to Nussbaum (loc. cit.) that the sperm passed through special ducts, but admitted that these were clearly connected with normal malpighian capsules in which both sperm and injection mass were to be found mixed. The rapid exchange of 'Aufsätze', 'Bemerkung über Aufsätze', &c., which followed between the two authors is not here cited, as it contributed nothing to the solution of the problem, ultimate agreement being reached that the difference in their results was a natural difference between *Rana fusca* and *Rana esculenta*.

This controversy led Beissner (1898) to investigate the kidneys of *Rana esculenta* and *Rana fusca*. He wrote a very short paper, nearly half of which is historical summary. This paper is illustrated with two admirable diagrams which showed that the differences between Frankl and Nussbaum could be reconciled if it were postulated that *Rana esculenta* had both a dorsal and a ventral transverse sperm-canal, while *Rana fusca* had only a dorsal one; if this were so, then sperm would have not only special canals (Frankl on *Rana fusca*) but they would pass through normal units (Nussbaum on *Rana esculenta*) in order to reach the second transverse canal of this latter form. As both his title and text show he never intended to do more than reconcile the two outstanding opinions.

Gaupp (1904), who was then preparing the new edition of Ecker's und Weidersheim's 'Anatomie des Frosches', republished both of Beissner's diagrams and accepted his postulate of a second transverse canal as an observed fact. The diagrams have since been widely published in text-books, and thus has arisen our present conception of the sperm-passage in *Rana*. Beissner's theoretical diagrams have even (Stewart 1927) been accepted as wax-reconstructions.

With Gaupp's publication (loc. cit.) the matter was taken as finally settled, if we except a brief statement by Gerhartz (1905) that Nussbaum's view was correct.

Interest arose again when the 'germ-track' controversy produced a spate of papers on the development of various gonads. In the course of a study of the development of the sex-cells of *Rana*, Kuschakewitsch (1910) makes some mention of the vasa efferentia. He considers that they arise from the testis-stroma at a time when this is still confused with the kidney blastema. Witschi (1913) gives an excellent account of the development of the collecting ducts within the testis and points out that these grow out from the central strand of stroma; they acquire a secondary connexion with the extra-testicular portion of the duct system. Swingle (1925) also discussed the development of the testis, but in referring to the development of the vasa efferentia he stated that everything was already known about it. He quoted Kuschakewitsch and Witschi (*loc. cit.*), attributing to the former the statement that tubules originating from the kidney end blindly before reaching the testis; I cannot find this stated in Kuschakewitsch (1910). Swingle's most remarkable suggestion, however, is that the indifferent gonad is induced into a testis by the arrival of the growing vasa efferentia; it is a little difficult to imagine what starts the vasa efferentia growing, or stops them growing and permits a female to be produced. Van Oordt (1922) in the course of completing Witschi's work on testis development, examined the genital connexion in some post-metamorphic *Rana fusca*; in a few examples he found ducts which originated from the kidney but did not reach the testis and also records the appearance of some unconnected ducts in the fat-bodies.

It is noteworthy that none of these investigators of the gonad traced the vasa efferentia into the kidney and none therefore can offer any suggestion as to the origin of the outgrowths which they presume.

The most recent contribution to the problem is that of Lloyd (1928), who points out that modern text-books of zoology state that the sperm passes through ordinary kidney-ducts, and says that he has examined many hundreds of sections without finding a sperm-duct. The work is cited here only as proof of the consensus of modern opinion.

## (ii) Origin.

There is no doubt that the rudiments of the vasa efferentia exist from the earliest appearance of the gonadic ridge. In Part I of the present investigation it was confirmed that the nephrotome of *Rana temporaria* breaks up into separate blastema cells at the time when the dorsal and ventral sheets of mesoderm separate. As the dorsal edge of the ventral sheet curves inwards, pushing the archinephric duct into its (larval) median position, these blastema cells come to lie in the retro-peritoneal connective tissue just on the outside of the duct, between this and the space which will be occupied by the gonad. There are, therefore, always a considerable number of blastema cells lying between the archinephric duct, round which the kidney will develop, and the genital ridge in which the gonad will develop. Fig. 20, Pl. 22, is a section through a 22-mm. tadpole, showing this appearance upon the right side. The large duct is the archinephric duct against the latero-dorsal wall of which a kidney unit (*rdu.*) is appearing. The rudiment of the gonad (*g.*) is clearly shown to be connected by a thick sheet of blastema with the main mass of the kidney blastema. The sheet here shown in section continues for the entire length of the gonad. It is important to notice at this point that the sheet runs over the median ventral wall of the large inter-renal vein (*irv.*). The vasa efferentia are produced by the segregation of this sheet into ducts, and there is no ground for regarding this tissue as an outgrowth from any part of the kidney, since nephrogenetic blastema cells are present before there is any differentiation of kidney units.

## (iii) Subsequent Development.

The subsequent development of the vasa efferentia is best described by tracing the early development backwards, and the late development forwards, from an intermediate position. The stage selected as intermediate is a 35-mm. frog; that is, one which is just entering its second year after leaving the water.

Fig. 4, Pl. 20, shows the relationship of vasa efferentia (*ve.*), testis (*t.*), and kidney. The straight tubule (*st.*) ends in an abortive unit,<sup>1</sup> the point of attachment being marked at *as.* An

<sup>1</sup> These 'abortive units'—so named in Part I of this investigation—are

excretory unit joins the straight tubule at *ae.* and the malpighian capsule (*mc.*) of this unit appears lower in the figure. The vas efferens (*ve.*) is not directly attached to the unit but terminates in the rudiment of Bidder's canal (*rb.*). It will be realized that the interconnexions of all these parts are rather complex and best studied in a reconstruction.

Fig. 9 *a* (Pl. 21) shows a reconstruction of this same region; at 9 *b* the reconstruction has been dissected. The straight tubule (*st.*) passes under the malpighian capsule (*mc.*), curves upwards towards the observer, and then passes to the right before looping across, down, and back to the malpighian capsule. There is no trace of any of the conventional tubule divisions of a normal unit. From the base of the loop indicated by *z* in fig. 9 *b*, Pl. 21, a solid strand of cells leaves the inner side of the middle loop. This passes out through the middle of the loop to the right and turns back (*ca.*) to become attached (at *az.*, seen in both 9 *a* and *b*) to an irregularly ovoid mass of blastema (*rb.*). A thick, irregular projection from the upper side of this mass of blastema curves upwards and over to narrow down as the vas efferens (*ve.*). It is obvious that both *ca.* and *ve.*, though they are distinct at this point, contribute to the adult vas efferens; and that *rb.* can only be explained as the rudiment of Bidder's canal. The arrangement shown in this reconstruction is found at the kidney end of every vas efferens which crosses laterally from the testis to the kidney. At the extreme anterior end of the testis, however, an altogether different arrangement prevails.

Fig. 29 (Pl. 23) is taken from the same series and is cut just anterior to the testis through about the middle of the fat-bodies (*fb.*). A duct (*ap.*), here just dividing into two, runs alongside and partially through the fat-bodies. Posteriorly to this level (fig. 28, Pl. 23) the two ducts enter the extreme anterior tip of the testis (*t.*), into which they pass, branching out (fig. 27, Pl. 23) as the internal sperm-collecting system of the testis. In this figure the darkly stained mass labelled *ap.* is

unquestionably the true sexual units of the frog's kidney. Each straight tubule ends in such a unit. I am not prepared to say how much of the straight tubule is homologous with the more posterior collecting-trunks and how much belongs to the functional tubule of this abortive sexual unit.

actually only one of the two ducts, the other lying outside the field of view. Two testis tubules, one labelled *tt.*, can be quite clearly seen to open into *ap.* As this anterior duct passes forwards from fig. 29, Pl. 23, it curves slowly and gradually round the dorsal side of the kidney and ultimately ends in a very much modified unit (*du.*, fig. 31, Pl. 23). In this section a normal abortive unit, belonging to the straight tubule (*st.*), is also shown.

The reconstruction in fig. 8, Pl. 21, which is to half the scale of figs. 9 *a* and *b*, covers the two anterior straight tubules from the same kidney. The reconstruction is viewed from the ventral aspect, and its antero-posterior axis has therefore been reversed to aid in the correlation with fig. 9; *st. 1* is therefore the most anterior straight tubule and *st. 2* the second. The first straight tubule carries at its end two quite irregular units whose malpighian capsules are shown at *mc. 1a.* and *mc. 1b.* The true terminal unit, which lies at the median angle of the kidney, is even more irregular than the sexual units described for Triton (Gray, 1933). It shows the same lateral outgrowth (*la.*) coming off close to the base which is itself greatly swollen, while there are other outgrowths occurring further along the tube. The terminal malpighian unit (*mc. 1a.*) is so small and badly developed that it cannot possibly subserve any function. This most anterior unit of the kidney has no sexual connexion. The second unit attached to *st. 1* runs directly in a posterior direction, bends down and then sharply up, the very narrow connexion with the malpighian capsule (*mc. 1b.*) coming off from the underside of the upward bend. From the posterior curve of this bend there runs backwards a twice bent tube (*ay.*) which curves towards the outer edge of the kidney, dips behind the malpighian capsule (*mc. 1b.*), and forms the attachment for the anterior prolongation (*ap.*) shown in figs. 27 to 29 on Pl. 23. A third malpighian capsule (*mc. 1c.*) and tubule are given off from the most posterior curve of *ay.* This completes the attachments of *st. 1*. *St. 2* carries at its end a heavily coiled mass of tubules which, however, lack any malpighian capsule. It is partially coiled, and is formed apparently of developing blastema tissue, which rather resembles a capsuloblast vesicle (Gray 1930, p. 544) and may represent the missing malpighian capsule of this unit.

It is apparent that all these irregular coils and lumps have little relation to a normal kidney unit, the functions of which they most certainly cannot carry out, and that the anterior prolongation, though its structure and affinities suggest a vas efferens, will never carry sperm. It is a well-known fact that kidney blastema cells, when cultured *in vitro* with connective tissue cells, form well-differentiated tubules. This anterior region represents a culture, not *in vitro* but *in vivo*. The straight tubules, which are found very early in development, are formed fairly normally, but the remaining blastema, left in an area which has no sexual or excretory use, merely forms tubules in an aimless manner.

From these reconstructions, however, there emerge the facts that, during the second year after metamorphosis:

(1) The vasa efferentia run from the kidney to the edge of the testis.

(2) Within the kidney the vasa efferentia end in a mass of blastema representing Bidder's canal which is itself directly attached to an abortive unit terminating a straight tubule.

(3) The sperm-collecting network within the kidney is not yet connected to the vasa efferentia but is connected to an anterior prolongation which runs through the fat-bodies to end in a remarkable mass of tubules at the extreme anterior end of the kidney.

We already know, however, from an examination of a 22-mm. stage that:

(4) The edge of the testis is connected to the kidney from the earliest stage where either is recognizable.

There remains only to trace the origin and fate of the anterior prolongation and the manner in which the solid kidney-testis connexion becomes broken up into vasa efferentia.

The sheet of blastema which, in the 22-mm. tadpole (fig. 20, Pl. 22), connects the gonad to the kidney lies on the surface of the inter-renal vein. This vein increases very rapidly both in size and length; the blastema cells do not increase in number. The natural result is that the sheet of tissue is broken into a number of irregular masses. It is common knowledge that the



number of vasa efferentia is variable, which would be highly improbable were each an outgrowth from a specialized unit.

This breaking up of a solid sheet leads to the condition shown in fig. 13, Pl. 22. The vas efferens appear as a solid rod of cells which ends blindly at the edge of the gonad, but runs back across the ventral surface of the kidney to the lateral edge. This is a section through the first vas efferens of a 32-mm. tadpole—that is, one with four legs apparent. Figs. 18, 19, and 12, Pl. 22, are from successively more anterior sections of the same series and show the condition of the anterior prolongation at this stage. In fig. 18, Pl. 22, *rve.* marks the rudiment of the vas efferens which, in this stage, is not yet divided. The strand of darkly staining cells marked *ap.* in this figure can be easily identified in fig. 19, Pl. 22, where it is passing from the fat-body rudiment to the surface of the inter-renal vein *irv.*, and followed forward to fig. 12, Pl. 22, where it terminates against the archinephric duct.

Now, reverting to fig. 13, Pl. 22, it will be seen that identical cells line the lumen of the gonad and are, at this stage, clearly making contact with the vas efferens.

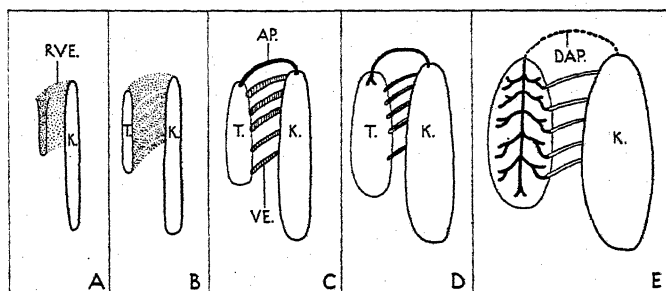
The process of the formation of the vasa efferentia up to this point is shown in Text-fig. 3. At A the testis is connected to the kidney by a solid sheet of tissue *rve.* In B this is breaking up posteriorly into vasa efferentia while the anterior portion of the sheet remains solid as the rudiment of the anterior prolongation. At the stage just described (32 mm.), represented by Text-fig. 3, C, the anterior prolongation is growing down through the testis to make a fresh contact with the vas efferens at the medial edge of the testis.

At a stage six weeks later than this the only changes in this arrangement have been produced by the further development of the kidney and testis. In fig. 21, Pl. 22, which is through a region analogous to fig. 13, Pl. 22, the kidney is now separating off from the inter-renal vein so that the renal veins are becoming more clearly marked; it will be noticed that the vas efferens runs along the peritoneal wall of one of these renal veins *y.* Figs. 14 and 22, Pl. 22, which compare with figs. 19 and 12, Pl. 22, show how the very rapid increase in size of the testis clearly marks

out the anterior prolongation. This condition persists to the end of the first year after metamorphosis.

The condition during the second year (Text-fig. 3, D) has been discussed above. The changes during the third year are only those which are required to bring the second-year condition to functional maturity.

The changes whereby the central strand, running caudad through the testis from the anterior prolongation, acquires



TEXT-FIG. 3.

Diagram to show the origin and growth of the vasa efferentia.

AP., anterior prolongation; DAP., degenerating anterior prolongation; K., kidney; RVE., solid sheet of tissue from which both AP. and VE. are produced; T., testis; VE., vasa efferentia.

connexions with the testis-tubules ('ampullae') has already been adequately described by Witschi (*loc. cit.*) and confirmed by Van Oordt (*loc. cit.*). The anterior prolongation itself (*ap.* fig. 32, Pl. 23) rapidly degenerates. Several writers (Van Oordt, Swingle) have reported disconnected lengths of tubule in the fat-bodies of post-metamorphic frogs and there is evidently considerable individual variation in the period at which degeneration takes place.

The vasa efferentia themselves (fig. 30, Pl. 23) do not alter in their renal attachments, though the straight tubules (*st.*, fig. 30, Pl. 23) are now much bent by the attachment of excretory units. It is interesting to note (fig. 16, Pl. 22) that even during the third year there is a close association between the point of entry of the vasa efferentia and the point of exit of the renal veins.

## D. DEVELOPMENT OF THE SEMINAL VESICLES.

## (i) Historical Note.

No work appears to have been carried out upon the development of the seminal vesicle. Both Gerhartz (1905) and Nussbaum (1912) studied the seasonal variation, the former from the physiological and the latter from the histological aspect. They both agree—as was, indeed, well known before them—that the adult vesicle is composed of many coiled tubes, but they offer no suggestion as to the origin. Gerhartz, throughout his paper, treats the seminal vesicles of *Rana* as homologous with the vas deferens of *Triton*, which also shows a seasonal variation. This view is untenable as the sperm-duct in *Triton* is quite definitely the remains of the archinephric duct, the appendages to this duct being true ureters, both in function and origin (Gray, 1932). The view that the seminal vesicle of *Rana* is homologous with the ureters of *Triton* is very attractive, since a morphological examination of the adult condition shows many points of resemblance. A study of the embryology, however, has convinced the writer that some other explanation must be sought and this explanation is put forward after the description which follows.

## (ii) Description.

The first trace of the seminal vesicles may be found in a four-legged tadpole. In this stage each of the archinephric ducts, when it leaves the kidney, passes ventrad along its own mesentery to enter the rectum. The general appearance is similar to the condition in *Triton* which was shown in Text-fig. 6, B, of Part II (Gray, 1932) of this investigation. In *Rana*, however, the mesentery is greatly expanded by large numbers of mesenchyme cells (c., fig. 26, Pl. 23) amongst which there lie from four to six agglomerations of kidney blastema (*rsvt.*). These agglomerations exactly resemble the capsuloblast vesicles which are still being formed in the kidney proper.

Each of these rudiments acquires a connexion with the archinephric duct shortly after metamorphosis, but undergoes no alteration at the end farthest from the duct.

During the course of the year following metamorphosis (fig. 25, Pl. 23) these rudiments elongate into short tubules, lying parallel with the archinephric duct and become furnished with lumina. There is, as yet, no branching or coiling, and this is well shown in the figure where one duct is seen sectioned through about the middle of its length, while the other is cut through its blind tip. The mesenchyme has developed into the plentiful connective tissue of which the greater part of the organ is formed.

During the second year (fig. 24, Pl. 23) there is none of the branching and coiling which will be required to increase the storage capacity of these tubules, but there is a considerable increase in the size compared to the length. This figure has been selected to parallel fig. 25, Pl. 23, as nearly as possible.

It is during the third year (fig. 23, Pl. 23) that the branchings and coiling typical of the adult make their appearance. Each tubule not only sends out numerous branches but also coils and twists round the other tubules.

Nussbaum's description of the histology of the adult seminal vesicle during resting period so thoroughly agrees with the appearance at this stage that no useful purpose would be served by a repetition.

### (iii) Possible Homologies.

The suggestion that the seminal vesicles of *Rana* are homologous with the ureters of *Triton* breaks down both on functional and embryological grounds. It has been shown (Gray, 1932) that the use of the word 'ureters' as applied to the excretory ducts of *Triton* is justifiable, as they are outgrowths of the archinephric duct. The seminal vesicles of *Rana* are derived from separate blastema masses and owe nothing of the material from which they are formed to the archinephric duct.

Two other hypotheses must therefore be considered. Either the vesicles are peculiar to *Anura* and were evolved independently in response to a functional necessity: or they may represent some of the ancestral kidney specialized to the function of sperm-storage. The present writer accepts the latter hypothesis in preference to the former, and it is not difficult to find a homology which would justify this acceptance.

It has been shown (Gray, 1930) that there arise throughout the whole length of the kidney a series of early units which degenerate throughout the length of the functional kidney, except for a slightly later generation which persist as straight tubules. The present investigation has shown that these straight tubules are all that remain of the sexual kidney shown more clearly in Triton. Now there is a marked similarity between the development of the straight tubule units and the tubules of the seminal vesicle. Each starts as a capsuloblast vesicle, delayed both in its origin and development; each is primarily devoted to a sexual function. It seems reasonable to suppose that at one time the sexual units extended throughout the whole length of the kidney and that they have been retained only at the anterior and posterior ends, in which regions alone does there exist any need for a sexually specialized unit.

The outstanding peculiarity in the development of the seminal vesicles is their origin from separate blastema condensations and not from the archinephric duct itself, which latter would be the normal thing to have happened in the course of a separate evolution.

#### E. REVIEW.

The facts brought forward in this paper do not justify a lengthy comparative discussion. Nothing is known about the post-metamorphic development of the kidney in any other amphibian, and the few fragmentary papers which have appeared on the later development of the kidneys in other groups offer no opportunities for comparison. The formation of the vasa efferentia, even though here fully described for the first time, shows no departure from what is already known for other groups of vertebrates. It has long been accepted that the testis-kidney connexion in birds and mammals is primitive, and not a secondary production of either organ.

The discussion of the development of the frog's kidney is of more interest. It is now perfectly clear that the sexual and asexual kidneys in Amphibia are distinct from each other in origin and become intermingled only in course of development.

The sexual kidney is represented by two sets of units, the

interval between the appearance of each set being directly correlated with the functional need for their production. The first set, termed 'early units' in the writer's previous paper, develops rapidly to a functional condition as the pronephric method of excretion becomes insufficient for the tadpole's needs. The second set is modified at the extreme anterior and posterior ends to sexual function; in the middle region it secondarily acquires an excretory use, and develops more slowly.

The functional kidney of the adult develops separately from units which, since the excretory needs of the young animal are amply covered by the pronephros and early units, do not become directly or immediately attached to the archinephric duct. These units do not appear at all at the extreme anterior end. In the antero-middle region they become connected to the straight tubules, which here carry both sperm and excretory products; in the postero-middle region they become attached to the straight tubules which here, being without sexual function, lose their identity; in the posterior region of the kidney, where all the early units have degenerated and no straight tubules are formed, the units of the functional kidney become attached to secondary collecting-trunks, derived as outgrowths from the archinephric duct and obviously homologous with the ureters of *Triton*. In fact, the only difference between *Rana* and *Triton* in this region is that the latter has the ureters, whose walls must therefore be furnished with some muscular support, outside the main mass of the kidney.

The whole course of the development of the kidney of the frog may be presented in tabular form:

### SEXUAL UNITS.

(Units of the first generation.)

#### A. EARLY SET. ('Early units', Gray, 1930.)

- |   |   |
|---|---|
| (1) Anterior Region . . . . .             | Few develop to a functional condition.  |
| (2) Middle and Posterior Region . . . . . | Develop rapidly to a functional condition, but degenerate at about the same time as the pronephros. |

## B. LATER SET. (Straight tubules.)

- |                                |  |
|--------------------------------|--|
| (1) Anterior Region . . .      | Persist as straight tubules which only carry sperm.  |
| (2) Antero-Middle Region . .   | Persist as straight tubules which carry both sperm and excretory products.                                       |
| (3) Postero-Middle Region . .  | Persist as straight tubules which carry only excretory products and finally lose their identity through coiling. |
| (4) Posterior Region . . .     | Never develop.   |
| (5) Extreme Posterior Region . | Form the tubules of the seminal vesicles.  |

## FUNCTIONAL UNITS OF ADULT KIDNEY.

- |                               |   |
|-------------------------------|---|
| (1) Anterior Region . . .     | Never develop, but are pushed into this region during the second and third years after metamorphosis. Are always connected to straight tubules of mid-region. |
| (2) Antero-Middle Region . .  | Form connexions to sperm-carrying straight tubules.   |
| (3) Postero-Middle Region . . | Form connexions to the most posterior of the straight tubules.  |
| (4) Posterior Region . . .    | Extensively developed during the second and third years after metamorphosis. Connected to branched and anastomizing outgrowths of the archinephric duct.      |

## ACKNOWLEDGEMENTS.

This research was originally suggested to me some years ago by Professor MacBride, but could not then be carried out owing to lack of material, and the present paper is the result of a request for information from Professor Hirsch of Utrecht. The work has been carried out in the Department of Zoology of Edinburgh University, and I am indebted to Professor Ashworth for his interest and encouragement. I have been greatly assisted by my wife, who summarized and translated some of the more voluminous of the early German writings on the kidney-testis connexion.

## F. SUMMARY

## The Post-Metamorphic Development of the Kidney.

1. The principal changes are in the method of attachment of malpighian unit to archinephric duct.

2. In the anterior region of the kidney there are few functional units attached to the straight tubules. (Text-fig. 1, A, C, F.)

3. In the middle region of the kidney there are many functional units attached to the straight tubules. (Text-fig. 1, B, D, G.)

4. Additional units are produced, after the end of the first year, only in the posterior region of the kidney.

5. There are no straight tubules in this newly formed posterior region so that the units become attached to outgrowths from the archinephric duct. (Text-fig. 1, E, H.)

6. These outgrowths, termed collecting-trunks, branch and anastomose.

7. Accessory peritoneal funnels are formed from blastema lying in that part of the kidney which adjoins the gonad. (Text-fig. 2, C.)

8. These funnels are furnished with longer tails than those previously produced. (Text-fig. 2, D.)

9. The tails are in close association with renal arterioles which subsequently pass to excretory units.

10. It is suggested that the arterial supply of the 'tail' may purify the coelomic fluid which the funnels return to the renal venules.

## The Development of the Vasa Efferentia.

11. The origin of our present conception of the formation of the vasa efferentia is traced to Gaupp's adoption of Beissner's compromise between the views of Hoffman and Spengel. (Pp. 454 to 458.)

12. The gonadic ridge is primitively connected to the region of the developing kidney by a sheet of kidney blastema. (Text-fig. 3, A.)

13. This sheet breaks up into (a) a series of rudimentary vasa



efferentia; (b) an anterior prolongation connecting the anterior end of the gonad to the anterior end of the kidney. (Text-fig. 3, B, C.)

14. The rudimentary vasa efferentia ends in a mass of blastema lying along the edge of the kidney.

15. This mass, from which Bidder's canal subsequently develops, is itself connected to the abortive unit which terminates the straight tubules. (Figs. 9 *a* and *b*, Pl. 21, Fig. 4, Pl. 20.)

16. The anterior prolongation ends in a mass of kidney tubules at the anterior end of the kidney. (Fig. 8, Pl. 21.)

17. It is suggested that these anterior units are without functional significance.

18. The testis end of the anterior prolongation grows downwards into the testis and becomes connected to the tubules and vasa efferentia. (Text-fig. 3, D.)

19. The kidney connexions of the vasa efferentia remain unaltered but the anterior prolongation degenerates (Text-fig. 3, E.)

#### The Development of the Seminal Vesicles.

20. In a four-legged tadpole there is a mass of mesenchyme, containing aggregations of kidney blastema, on the side of the archinephric duct between the points where this leaves the kidney and enters the rectum. (Fig. 26, Pl. 23.)

21. The mesenchyme forms connective tissue and the blastema aggregations form short tubules.

22. During the third year after metamorphosis these tubules branch and coil.

23. It is emphasized that the seminal vesicle derives nothing from the archinephric duct, and suggested that it may represent the remnants of the most posterior units of the sexual kidney.

#### Review.

24. The author's views on the relationships of the various units found in the developing kidney of *Rana* are expressed in tabular form on pp. 467 and 468.

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## EXPLANATION OF PLATES 20 TO 23.

The magnifications given are of the figures as reproduced.

## LIST OF COMMON ABBREVIATIONS.

*a.*, artery; *ad.*, archinephric duct; *ae.*, attachment of excretory unit to *st.*; *ap.*, anterior prolongation; *as.*, attachment of *au.* to *st.*; *au.*, abortive unit; *ay.*, attachment of *ap.* to *du.*; *ax.*, attachment of *au.* to *rb.*; *b.*, blastema; *bt.*, blindly ending tubule; *c.*, connective tissue; *ca.*, connexion of Bidder's canal to *au.*; *ct.*, collecting-trunk; *dm.*, developing *mc.*; *du.*, dorsal abortive unit; *fb.*, fat-bodies; *g.*, gonad; *k.*, kidney; *irv.*, interrenal vein; *la.*, lateral outgrowth; *mc.*, malpighian capsule; *pcv.*, posterior cardinal vein; *pf.*, peritoneal funnel; *ra.*, renal artery; *rat.*, renal arteriole; *rb.*, rudiment of Bidder's canal; *rdv.*, rudiment of *du.* to which *ap.* becomes attached; *rsvt.*, rudiment of *svt.*; *rv.*, renal venule; *rve.*, the solid sheet of tissue from which the vasa efferentia are derived; *st.*, straight tubule; *sv.*, seminal vesicle; *svt.*, tubule of seminal vesicle; *t.*, testis; *tt.*, testis tubule; *ve.*, vas efferens; *x.*, tubules without attachment or apparent function; *y.*, renal vein; *z.*, looped tubule connecting *mc.* to *st.*

## PLATE 20.

Microphotographs showing the general structure of the post-metamorphic kidney in transverse section.

- Fig. 1.—Postero-middle region of a third-year (53 mm.) frog.  $\times 75$ .  
 Fig. 2.—Posterior region of a third-year (53 mm.) frog.  $\times 75$ .  
 Fig. 3.—Middle region of late second-year (42 mm.) frog.  $\times 75$ .  
 Fig. 4.—Sexual region of early second-year (35 mm.) frog.  $\times 150$ .  
 Fig. 5.—Middle region of third-year (53 mm.) frog.  $\times 50$ .  
 Fig. 6.—Sexual region of early second-year (35 mm.) frog.  $\times 50$ .  
 Fig. 7.—Extreme anterior region of early second-year (35 mm.) frog.  $\times 80$ . *Erratum for av read au.*

## PLATE 21.

Graphic reconstructions to show the relationships of the vasa efferentia and anterior prolongation in an early second-year (35 mm.) frog.

- Fig. 8.—Anterior end of kidney from dorsal aspect. Anterior end to left of plate.  
 Fig. 9.—Region of second vas deferens. Anterior end to right of plate.  
*a.* Reconstruction from ventral aspect.  
*b.* The same with the medial portion of *ve.* and the central portion of *rb.* removed, to show the origin of *ca.* from the looped tube *lt.* connecting *mc.* to *st.*

## PLATE 22.

Microphotographsshowing the structure and relationships of the accessory peritoneal funnels (figs. 10 and 15), the anterior prolongations (figs. 12, 14, 18, 19, 20, 22), and the vasa efferentia (figs. 11, 13, 16, 21).

- Fig. 10.—Late second-year (42 mm.) frog.  $\times 175$ .

- Fig. 11.—Newly metamorphosed frog.  $\times 100$ .  
Figs. 12 and 13.—Four-legged (32 mm.) tadpole.  $\times 175$ .  
Fig. 14.—Newly metamorphosed frog.  $\times 100$ .  
Fig. 15.—Third-year (53 mm.) frog.  $\times 175$ .  
Fig. 16.—Third-year (53 mm.) frog.  $\times 3$ .  
Fig. 17.—Central portion of fig. 5.  $\times 200$ .  
Figs. 18 and 19.—Four-legged (32 mm.) tadpole.  $\times 175$ .  
Fig. 20.—22-mm. tadpole.  $\times 175$ .  
Figs. 21 and 22.—Newly metamorphosed frog.  $\times 100$ .

## PLATE 23.

Microphotographs to show the structure and relationships of the vasa efferentia (fig. 30), anterior prolongation (figs. 27, 28, 29, 31, 32), and seminal vesicles (figs. 23–6).

- Fig. 23.—Seminal vesicle of third-year (53 mm.) frog.  $\times 45$ .  
Fig. 24.—Seminal vesicle of late second-year (42 mm.) frog.  $\times 80$ .  
Fig. 25.—Seminal vesicle of early second-year (35 mm.) frog.  $\times 200$ .  
Fig. 26.—Seminal vesicle of four-legged (32 mm.) tadpole.  $\times 300$ .  
Figs. 27–9.—Early second-year (35 mm.) frog.  $\times 150$ .  
Fig. 30.—Late second-year (42 mm.) frog.  $\times 60$ .  
Fig. 31.—Early second-year (35 mm.) frog.  $\times 150$ .  
Fig. 32.—Late second-year (42 mm.) frog.  $\times 100$ .

The measurements given for frogs are taken with a flexible measure from the tip of the snout to the anterior margin of the cloaca. Those for tadpoles are projected measurements from the tip of the snout to the end of the tail.

## Notes on orientation.

## PLATE 20.

Figs. 1, 2, 3: lateral to right; ventral to bottom; figs. 4 and 5: lateral to right, dorsal to bottom; fig. 6: lateral to bottom, dorsal to left; fig. 7: lateral to bottom, dorsal to right.

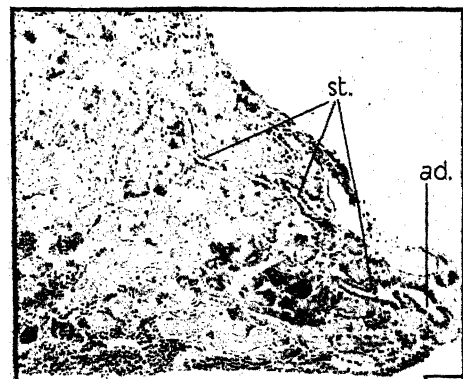
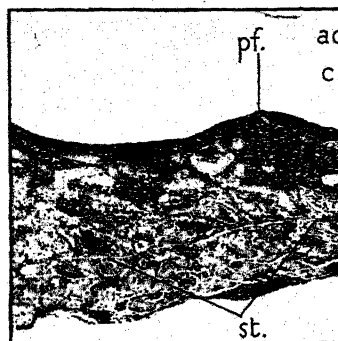
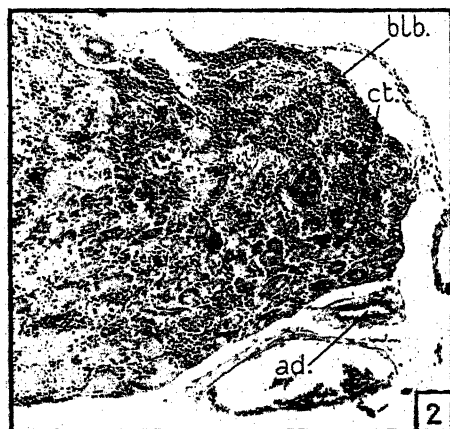
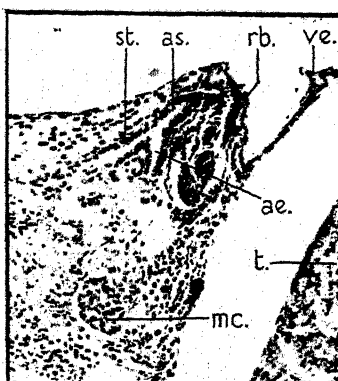
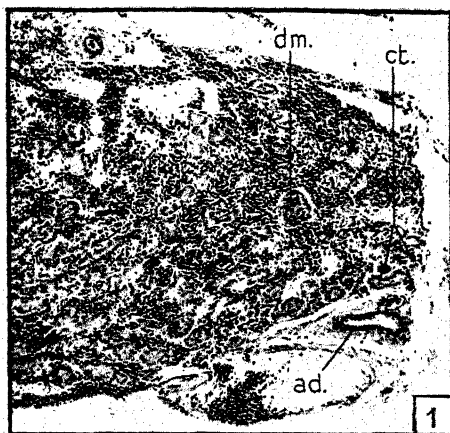
## PLATE 22.

Figs. 11–14 and 18–22: The testis or anterior prolongation lies medio-ventral to the kidney or archinephric duct. Figs. 10 and 15: peritoneal surface of kidney to top.

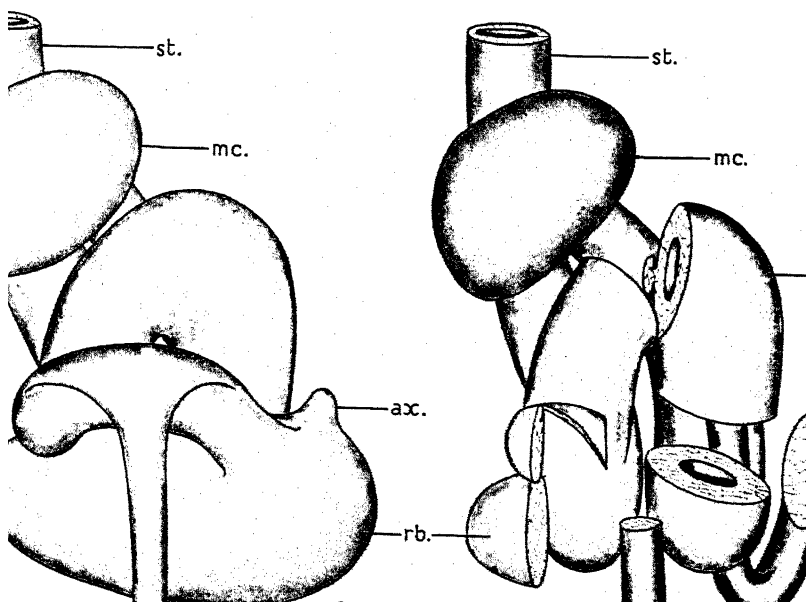
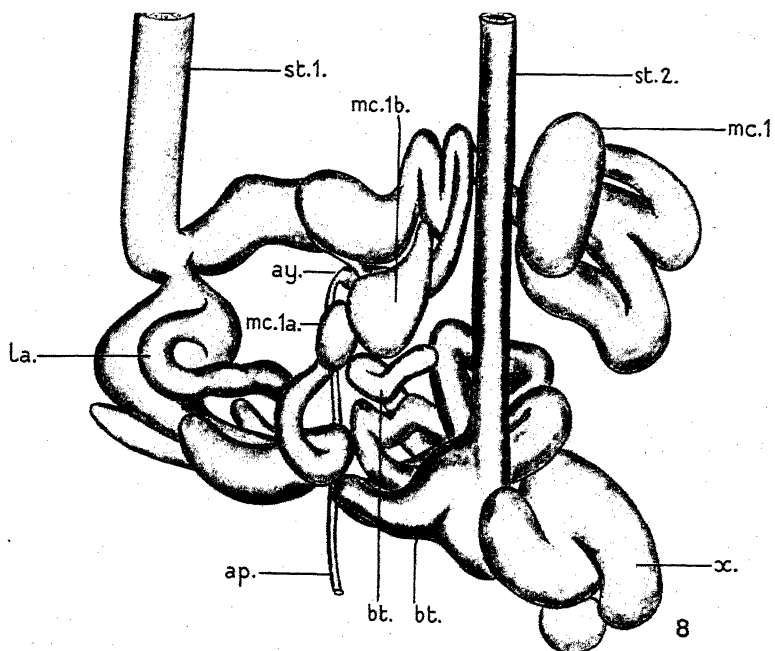
## PLATE 23.

Figs. 27–9, as figs. 11–14, &c., in Pl. 22. Figs. 30 and 32: lateral to bottom, ventral to left. Fig. 31: median to bottom, ventral to right.



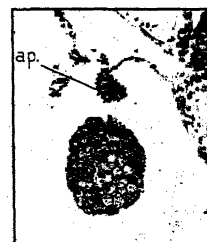
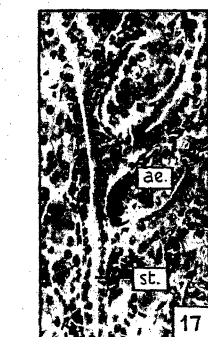
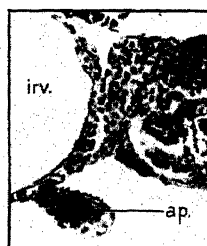
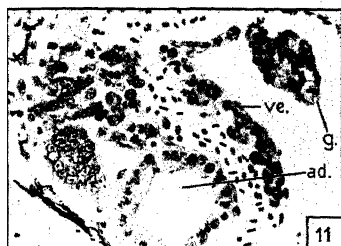
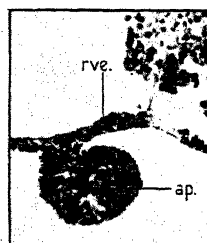
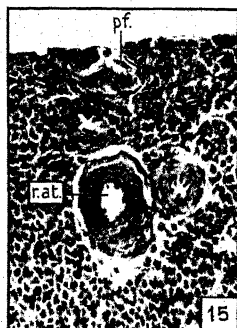




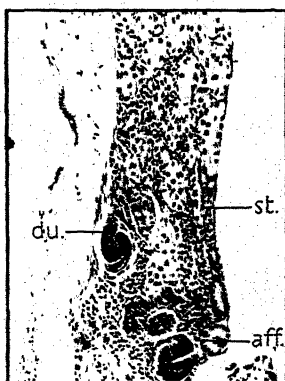
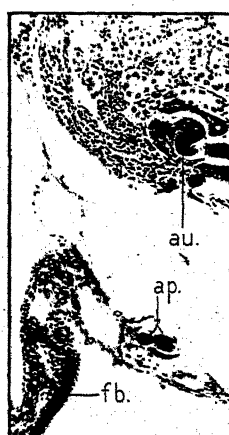
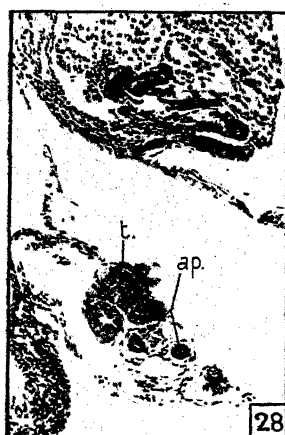
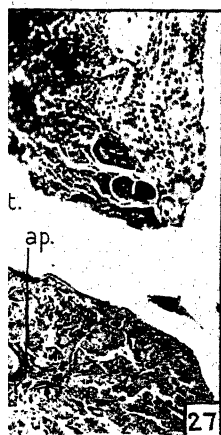
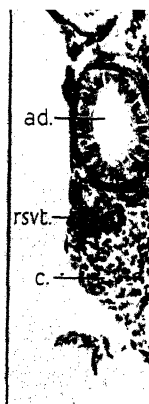
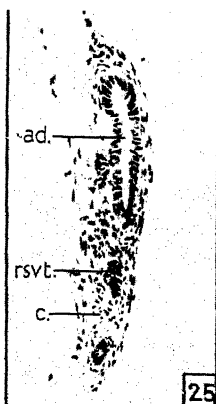
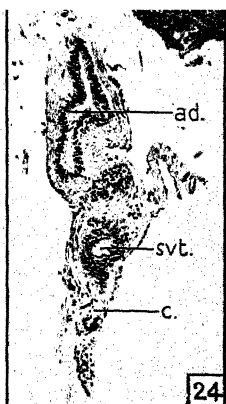
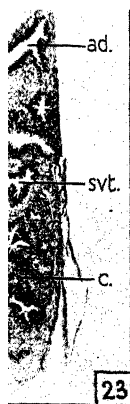














# The Innervation of the Adrenal Glands of Mammals; a Contribution to the Study of Nerve-endings.

By

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Department of Zoology and Comparative Anatomy, University Museum,  
Oxford.

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With Plate 24.

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## INTRODUCTION.

THE nature of the mechanism by which nerve impulses in one cell stimulate other cells to activity still remains a matter of considerable controversy, and the present study is an attempt to further our knowledge of the subject by an accurate study of the innervation of the adrenal glands.

Certain points in the general anatomical arrangement are almost unanimously accepted, namely, that the nerve-fibres from the sympathetic chain form a plexus on the capsule. From this plexus several nerve-bundles pass in through the cortex to the medulla, where the nerve-fibres branch profusely among the chromophil cells.

There is considerable doubt as to whether any of the nerve-fibres actually end in the cortex. Collateral branches are given off as the bundles pass through the cortex, and Dogiel (1894) and Fusari (1891) in adult animals, and Brauer (1932) in early

embryonic forms of the chicken, describe nerves branching around cortical cells. Alpert (1931) saw the fibres ending within the cortical cell. On the other hand, Giacomini (1897) found very few nerves in the cortex of birds, and, in Selachians, Young (1933) could find no nerves in the interrenal except those to the blood-vessels.

As regards the innervation of the medullary tissue, the first question is whether the fibres are preganglionic as suggested by Elliott (1913). Young (1933) showed that in Selachians some at least of the motor fibres to the chromophil cells came from postganglionic nerve-cells lying in or near the suprarenal tissue, and that therefore in this case Elliott's hypothesis could not be altogether correct. It was hoped that further study of the intramedullary nerve-cells in mammals would throw more light on this question which is of some considerable interest in view of the different methods of action of pre- and postganglionic synapses (see Feldberg et al., 1934).

Perhaps the most interesting problem of all is the relation of the nerve-fibres in the medulla to the secretory cells. Fusari (1891) described a nerve net around groups of chromophil cells. He saw terminations ending in plaques or discoidal knobs, but contended that these were artifacts. Similar nerve nets around individual cells were found by Dogiel (1894).

On the other hand, Alpert (1931) describes the nerve-fibres to both cortical and medullary cells as penetrating within the cell 'either by a short straight twig or a delicately curving fibril which tapers down to end near the nucleus'.

There has been a similar difference of opinion as regards the innervation of other glands. Intercellular endings have been found by Cajal (1891) in the pancreas and salivary glands; by Arnstein (1895) in several glands; by von Greving (1934) in the pancreas; and by Stormont (1928) in the salivary and thyroid glands. Intracellular endings are reported by Kolmer (1905) in the dermal and lacrimal glands of *Triton cristatus*; by Tricomini-Allegro (1903-4) in the mammary gland; by Puglisi-Allegro (1904) in the lacrimal gland; and by Kubo (1933) in the liver. The general conclusion that the end organs lie within the cytoplasm is in line with the work of Boeke (1932) on nerve-

endings in muscles, whereas the opposite conclusions have been reached among others by Hoff (1932), who believes that the terminal boutons seen in the spinal cord are merely in contact with the dendrites which they stimulate, and that there is no neurofibrillar continuity such as has been suggested by so many workers (see Tiegs, 1931).

There is, however, another possibility, namely, that the nerve-fibres passing close to the secretory cells can stimulate them without ending either within them or on their surface. That such stimulation does in fact occur is suggested by the whirls of fibres found particularly around sympathetic neurons (see Young, 1933).

#### STATEMENT OF PROBLEMS.

There are therefore five problems which have been dealt with during the present work.

1. To discover whether the nerve-fibres in the cortex innervate the secretory cells or merely pass through with the blood-vessels.
2. To determine whether there is any postganglionic innervation of the chromophil cells from intra-medullary neurons.
3. To discover whether the finest branches of the nerves in the medulla penetrate inside the cells which they innervate.
4. To find out whether the secretory cells are always innervated by terminal branches of the nerve-fibres, or whether the latter may deliver a stimulus as they pass among the cells.

#### MATERIAL AND TECHNIQUE.

For the principal study of the adrenal gland, the guinea-pig was used as representative of mammals. The male sex was uniformly chosen in the case of adults. For purposes of general comparison several embryonic stages (about 28 and 50 days) and a series of young animals of both sexes (1 and 2 hours, 2½, 4, 7, 9, 12, 14, 16, 18, and 21 days) were examined; also suprarenal glands from other mammals, rabbit and mouse. All the animals were killed by a blow on the head and dissected immediately afterwards.

Greatest success in the fixing and staining of the nerve-fibres was obtained with Cajal's chloral hydrate method.



## (1) Fix for 24 hours in:

Chloral hydrate . . . . .	2.5 gm.
95 per cent. alcohol . . . . .	40 c.c.
Distilled water . . . . .	40 c.c.
Pyridine . . . . .	20 c.c.

(2) Wash in distilled water until little or no smell of pyridine remains and transfer to 97 per cent. alcohol for 24 hours.

## (3) Wash in distilled water.

(4) Place in 2.5 per cent. silver nitrate at 37° C. 9-12 days was found to be the most satisfactory length of time. The longer times being better for nerve-cells.

## (5) Short wash in distilled water (1 minute sufficient).

## (6) Reduction 12-24 hours in

Hydroquinone or pyrogallol acid . . . . .	1 gm.
Neutral formol . . . . .	10 c.c.
Distilled water . . . . .	90 c.c.

(Hydroquinone gave clearer results.)

(7) Dehydrate rapidly, embed in paraffin wax, and section 15-30  $\mu$  thick.

On some occasions the nuclei of these sections were stained with toluidin blue. This, however, was found to be unnecessary. The above method gave clearly fixed cells and nuclei, whereas the stain rendered indistinct the contrast between the black nerve-fibres and the yellow cytoplasmic background.

#### INNERVATION OF THE ADRENAL CORTEX.

The general arrangement of the nerves to the adrenal were found to be as described by other workers, namely, that nerve-bundles pass off from the plexuses in the capsule, through the cortex to the medulla (fig. 1, Pl. 24). Smaller branches are given off from the bundle as they pass through the cortex and some of these seem to pass into the cortical tissue itself and to end around the cells of the latter. In the cavy no nerve-endings were distinguished in this region, but only swellings on the course of the fibres as they pass the cell (*boutons de passage*). However, in the mouse both types of *boutons* were found. These *boutons* always seemed to lie on the surface of the cortical cells, never within them (fig. 2, Pl. 24).

There is, therefore, some evidence that the cells of the cortex may be directly innervated. However, it must be stressed that the cortex, in general, is exceedingly poor in nerve-fibres in comparison with the medulla. Even if the boutons described above do represent motor endings, yet it is not suggested that the majority or even a large proportion of the cortical cells are controlled in this way.

No nerve-cells were found in the cortex of young or adult guinea-pigs or mice, though Kubo (1934) has recently demonstrated their existence in man.

#### OCCURRENCE OF NERVE-CELLS IN THE ADRENAL MEDULLA.

It has not been possible during the present investigation to settle definitely the question of the pre- or postganglionic origin of the nerve-fibres in the medulla. However, some evidence on this point is provided by the finding in the medulla of the younger forms of many nerve-cells, often as many as 20-25 in one section, 25  $\mu$  thick. Very few, however, are seen in the adult forms, in which they stain very indistinctly. This great difficulty in staining may account for their apparent rarity.

The author endeavoured to discover whether there is direct innervation of the chromophil cell from the intramedullary nerve-cell in the guinea-pig, but unfortunately either the nerve-fibres were cut too short by sectioning or the nerve-fibres from the nerve-cell were too complex to differentiate the course of separate nerves. However, Mr. Young kindly let me examine his sections of suprarenal tissue in Selachians, and several examples were found (fig. 3, Pl. 24). Whereas it cannot be concluded from this that a similar situation exists in the two animals, yet the observation suggests that at least some of the nerves in the medulla are postganglionic.

#### INNERVATION OF THE ADRENAL MEDULLA.

The bundles of fibres which enter the medulla break up to form plexuses around the lobules into which the chromophil tissue is divided. From these plexuses smaller fibres run in and out among the cells of the lobule, forming a very complete network, such that in the adult every chromophil cell is in contact

at least along one side with a nerve-fibre (fig. 4, Pl. 24). At intervals along the fibres there are swellings, boutons de passage, whose significance is discussed below, and some of the fibres come to an end as a terminal bouton (fig. 5, Pl. 24). A very careful examination has been made to discover whether either the fibres or the boutons penetrate into the cytoplasm of the chromophil tissue. This question is by no means easy to decide, but study of the preparations has led me to conclude that no such penetration occurs and that the nerve-fibres and boutons always lie on the surface of the chromophil cell, never within it.

Boutons de passage and boutons terminaux were present in the medulla of all adult animals examined, and in the later embryonic and young stages of guinea-pigs. However, even among adults of the same species there is no uniformity of distribution, for in many forms the boutons are concentrated in certain limited areas, whereas in other individuals they are scattered throughout the gland.

The boutons also vary in size, especially the boutons de passage (fig. 6, Pl. 24). Their exact structure is difficult to judge because of the many variations. Often the endings appear as hollow loops (fig. 6, Pl. 24) or oval bulbs either faintly (fig. 5, Pl. 24) or definitely (fig. 5, Pl. 24) fibrillar. Often, too, the nerve-fibres end in a slightly open fibrillar structure resembling a paint-brush (fig. 6, Pl. 24). Boutons de passage have an even more varied appearance: a hollow oval loop (fig. 6, Pl. 24); a loop with the nerve-fibre continuing through it (fig. 7, Pl. 24); or swellings composed of straight distinct fibrils (fig. 7, Pl. 24) or seemingly knotted and unevenly arranged fibrils (fig. 5, Pl. 24).

Considering the appearance of the structure of these boutons and the difficulty of staining them without altering their true nature, the author is unable to decide whether such neurofibrils in the boutons de passage and terminaux represent truly the living condition.

Previous investigators, who have been mentioned above, have stated that the boutons de passage and boutons terminaux act as synapses. This may be true, but there is another point to consider, namely, whether such boutons

represent the chief or only method of excitation, and what may be their importance in the innervation of the gland relative to the contacts made by the nerve-fibres which run so close to the surface of the secretory cells. An examination of a series of equal areas of medullary tissue was therefore made in 1 and 2 hour, 2½ and 4 days, and adult forms of the guinea-pig, both kinds of boutons and the medullary cells being counted, so as to enable the number of boutons per fifty medullary cells to be computed (see Table I). It is difficult to draw statistically exact

TABLE I  
Summary Table of Number of Boutons.

<i>Age of animal.</i>	<i>No. of fields of 50 μ sq. examined.</i>	<i>No. of boutons de passage per 50 chromophil cells.</i>			<i>No. of boutons terminaux per 50 chromophil cells.</i>			<i>Total average.</i>
		<i>Max.</i>	<i>Min.</i>	<i>Aver.</i>	<i>Max.</i>	<i>Min.</i>	<i>Aver.</i>	
1 hour	20	10.6	0	3.7	2.7	0	0.9	2.3
2 hours	20	5.0	0	2.1	1.4	0	0.4	1.2
2½ days	20	6.2	0	2.2	2.5	0	1.2	1.7
4 days	5	5.6	2	3.6	1.4	0	0.6	2.1
Average of above 4 animals	65	10.6	0	2.9	2.7	0	0.9	1.8
Adult	20	3.5	0	2.6	1.8	0	0.8	1.4

conclusions from such an investigation for there is always the possibility that the groups of microscopic fields may not have been in equal degree random selections from the sections, and that these selections may not have been representative of all the variations in an entire medulla. However, the arithmetical means of a series of twenty counts gives the best estimate obtainable in practice.

The data are not adequate to show statistically the relative number of boutons in animals of different ages, but in general there seem to be rather fewer boutons in the older animals. This is interesting when compared with Windle's (1930) investigation of the spinal grey matter of kittens. He found no boutons appearing until the kitten was 21 days old, and then only boutons de passage, the number increasing with

age. In the adrenal gland both types are found fairly early in embryonic life.

In comparing the average number of boutons de passage with that of boutons terminaux the results show the former to be  $3\frac{1}{2}$  times more numerous than the latter. This would suggest that the boutons de passage may be the more important. However, it is in considering the total number of boutons per fifty medullary cells that the results are most interesting.

There was considerable variation in the figures between different areas, the number of terminal boutons varying in the young animals from 0 to 2.7, in the adult from 0 to 1.8, and of the boutons de passage in the young from 0 to 10.6, and in the adult from 0 to 3.5. The average total number of boutons per fifty cells in the young forms was 1.8, and in the adult 1.4. In view of the smallness of these figures it seems certain that the boutons are not the main agent for stimulating the secretory cells, and in view of this it does not seem profitable to give any more detailed estimates of the variation between areas.

Mr. P. Heusner has been kind enough to let me mention, in this connexion, the work which he is now carrying out at Oxford on the superior cervical ganglion of cats. He has concluded that the boutons terminaux, if they exist, are too few in number to be of much significance, and that the close proximity of the finest preganglionic elements with the dendrites and the perikaryon of the postganglionic elements constitute the chief site of functional communication.

One can conclude from the above numbers that some other structure must aid in innervating these medullary cells. As has already been suggested it is quite possible that the nerve-fibres which form complex nerve nets throughout the medulla perform that function by stimulating the cell through contact with its surface. Such a method of stimulation has already been suggested for sympathetic ganglia (see Young, 1933), and would be in accordance with what we know of the general characteristics of secretory response, namely, the synchronous discharge of large numbers of cells. Such a type of innervation would

clearly be less suitable for a tissue such as striated muscle in which the necessary correlation requires the division of tissue into separate functional units. Even in a gland such as the adrenal, one would expect that gradation in activity would be brought about by activation of larger or smaller parts of the gland, and it is most unlikely that the terminal plexuses among the chromophil cells constitute a continuous network in the sense understood by Stöhr (1928) and others, which, on the current theories of nervous activity, would imply that all parts of the medulla would necessarily be activated together. In fact the occurrence of terminal boutons, even if they are rare, shows that sooner or later the fibres do come to an end.

#### SUMMARY.

1. The cells of the adrenal cortex of the cavy and mouse are sometimes innervated directly by nerve-fibres passing over and around the cells, but such nerve-fibres are very scarce in the cortex, the majority of whose cells are probably not under nervous influence.

2. There is a complex plexus of nerve-fibres among the cells of the adrenal medulla, the fibres of the plexus coming into close contact with the surface of the chromophil cells, but never penetrating inside them.

3. Formations comparable with the boutons de passage and the boutons terminaux of the central nervous system occur in this plexus.

4. However, the average number of boutons of both sorts per fifty medullary cells was found to be only 1·8 in young guinea-pigs, and 1·4 in adults. It is, therefore, concluded that they do not represent the main agent by which the secretory cells are activated, but that this function is performed by the nervous impulse passing in the network of fibres around the cell.

I am very grateful to Professor E. S. Goodrich and Mr. J. Z. Young for the suggestion of this problem, and their advice and assistance throughout the year. The work has been carried on in the Department of Zoology and Comparative Anatomy at the University Museum, Oxford.

## EXPLANATION OF PLATE 24

All figures are from preparations made with Cajal's method as described. Drawings were made with Zeiss camera lucida, 4 compensating ocular, and oil-immersion objective.

## KEY TO LETTERING.

*bp.*, bouton de passage; *bpm.*, bouton de passage with nerve-fibre passing through; *bt.*, bouton terminal; *c.*, cortex; *cap.*, capsule; *cbp.*, clearly fibrillar bouton de passage; *cbt.*, clearly fibrillar bouton terminal; *fbp.*, faintly fibrillar bouton de passage; *fbt.*, faintly fibrillar bouton terminal; *hbp.*, hollow loop bouton de passage; *hpt.*, hollow bouton terminal; *l.*, lobule; *m.*, medulla; *n.*, nerve; *nb.*, nerve-bundle; *nc.*, nerve-cell; *nf.*, nerve-fibrils; *pbt.*, paint-brush bouton terminal.

Fig. 1.—Photograph of section of adrenal gland in young guinea-pig showing general arrangement of nerves.

Fig. 2.—Section of the cortex next to capsule in a fourteen day old guinea-pig, showing nerve-fibres, several with boutons de passage, innervating the cortical cells.

Fig. 3.—Innervation of medullary cells in Selachians.

Fig. 4.—Detail of one medullary lobule in adult guinea-pig showing complex nervous plexus.

Fig. 5.—Drawing from a two and a half day guinea-pig showing large boutons de passage and smaller boutons terminaux.

Fig. 6.—Group of medullary cells in about a fifty day guinea-pig embryo showing different size boutons de passage and boutons terminaux. Also one ending resembling a paint-brush.

Fig. 7.—Group of medullary cells in an adult guinea-pig showing three different types of boutons de passage.

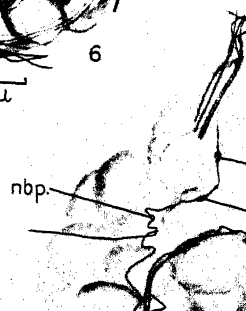
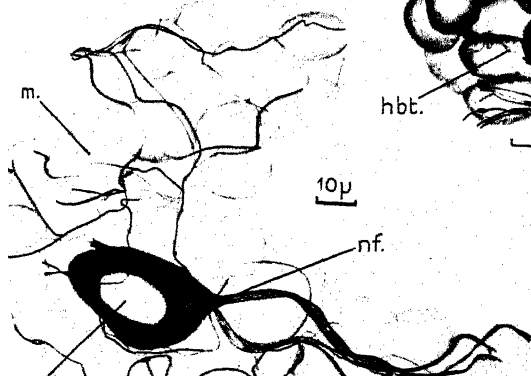
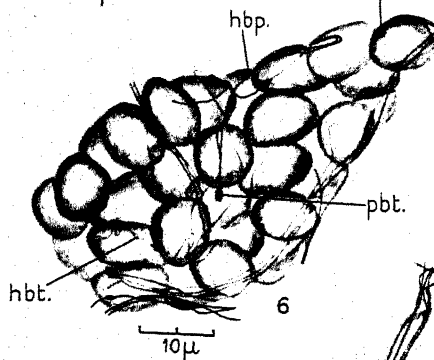
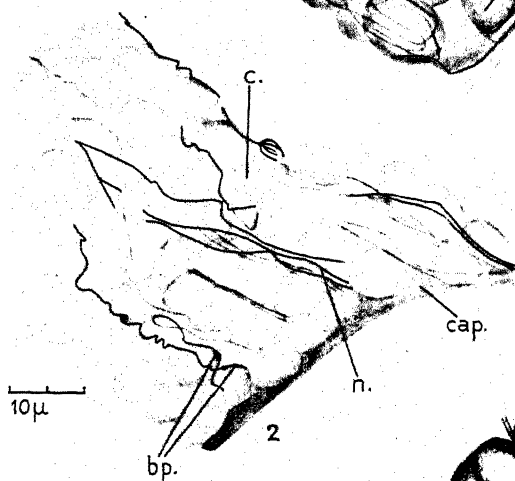
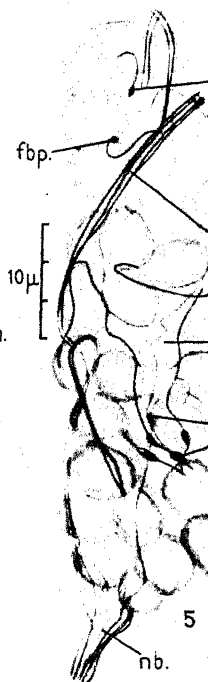
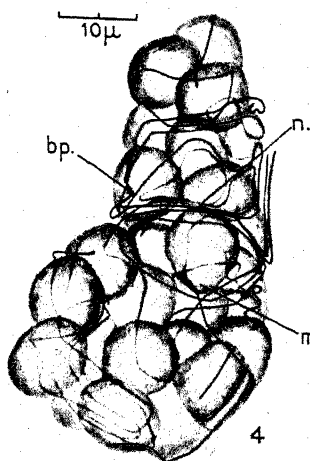
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# The Embryonic Development of the Stick-Insect, *Carausius morosus*.

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With Plates 25 and 26.

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## I. INTRODUCTION.

THIS paper is a contribution to our knowledge of the development of the germ layers in the stick-insect (*Carausius morosus* Br.).

The problem was suggested to me by Professor E. W. MacBride, who thought that in view of the contradictory statements that had been made in recent years concerning the formation of the germ layers in insects, a revision of the whole subject was called for.

(a) Material.—I was fortunate enough to obtain an abundant and regular supply of eggs of the Phasmid, *Carausius* (*Dixipus*) *morosus*, Br., the stick-insect of South India, from Miss D. Sladden, D.I.C., of the Imperial College of Science and Technology, who was breeding them in large numbers in order to investigate the inheritance of some of their characters. *Carausius* is a parthenogenetic insect belonging to the Orthoptera.

The eggs of *Carausius morosus* are very large, measuring about  $5 \times 3$  mm. They develop parthenogenetically and hatching takes place in about 90–120 days after dehiscence from the ovary, the rate of development varying greatly and depending on the temperature. Eggs kept at ordinary room temperatures in the summer show so great differences in the rate of development that the eggs of a given age may be at very different stages of development. The egg is enclosed in a hard chitinous capsule

<sup>1</sup> We regret to announce that the author died on May 15th, 1934, shortly after the completion of this paper.

showing the place of its previous attachment to the ovarian tubule by a ridge on the ventral side. At the anterior pole is a cap or operculum, attached to the capsule by serrated teeth. A pale rounded prominence is found on this cap. Inside the capsule there is a thin but almost impermeable membrane which is the chorion.

(b) Technique.—Owing to the large amount of yolk present double embedding in celloidin and paraffin was necessary. The method followed was mostly Newth's (1919) with modifications.

The eggs were placed in diaphanol for a few minutes to soften the chitin and the caps were removed. After washing in 63 per cent. alcohol in order to remove the diaphanol the eggs were fixed in hot (65°–70° C.) Bouin's picro-formol with a few crystals of urea and kept in this fluid for 10–12 hours; the chorion was pierced with a needle some time after the fluid had cooled. Slight shrinkage of the chorion resulted from this treatment and this enabled the eggs to be dissected out without suffering any damage. They were washed in 70 per cent. alcohol for at least 24 hours, and stored in 80 per cent. alcohol with a few drops of glycerine.

After the chorion had been removed the embryonic part of the egg was cut out, in order to facilitate the penetration of the celloidin solution, and stained in light green, then immersed in a mixture of equal parts of ether and alcohol for about 15 minutes, and transferred with a few drops of ether and alcohol to the top of a thick solution of celloidin (8 per cent.) in a deep dish. Gradual mixing took place and the material slowly sank into the celloidin. After at least 36 hours it was hardened in vapour of chloroform for 3–4 hours, and the embryo was cut out of the mass with just a little celloidin adhering to it, cleared in liquid chloroform, and re-embedded for 40–60 min. in paraffin (56° C.). Light green helps in orientating the embryo and can be easily washed out of the sections on the slides. The curvature of the posterior end of the embryo makes it impossible to obtain a complete series of either sagittal or transverse sections from the same embryo.

Sections 6, 7, or 8  $\mu$  in thickness were treated as follows:

floated in a film of water on the slide, celloidin softened in vapour of ether and alcohol, water removed, stretched and fixed, paraffin dissolved in xylol, placed for less than 40 seconds in oil of cloves to dissolve most of the celloidin, transferred to 90 per cent. alcohol in which the residual celloidin was hardened to form an adhesive film, stained, mostly in Ehrlich's or Delafield's haematoxylin, and counterstained in eosin. Early stages were stained in Heidenhain's iron haematoxylin in order to bring out the nuclear structure. Double staining in borax carmine and picro-nigrosin gave very satisfactory results for later stages of development. To obtain whole mounts the eggs were stained in bulk in borax carmine.

This work was carried out in the Huxley Research Laboratory of the Imperial College of Science and Technology at the suggestion and under the direction of Professor E. W. MacBride, F.R.S., to whom I wish to express my deepest gratitude for the very great help and encouragement he has given me throughout the whole period of my work. I am deeply indebted to Miss D. Sladden for supplying the material and to Mr. H. R. Hewer for the suggestions and criticisms he has always willingly given me in the preparation of this paper.

## II. ORGANIZATION OF THE EGG.

Sections of the ovarian tubules show the eggs in various stages of development. The maturation is immediately followed by the formation of the cleavage nucleus, its division, and the formation of the blastoderm.

In the youngest stage (fig. 1, Pl. 25) the egg is 5 mm. in length and 3.7-4.0 mm. wide. The nucleus is in the centre of the egg and surrounded by cytoplasm. It measures  $22\ \mu \times 18\ \mu$ . The anterior end of the egg (i.e. the end lying towards the anterior end of the ovarian tubule) is conical; the cytoplasm is undifferentiated and homogeneous.

When development begins the cytoplasm greatly increases in bulk, the nucleus being still in the centre. In transverse sections the shape is roughly polygonal. The cytoplasm is still homogeneous, but the part near the nucleus is clearer than the peripheral part. In longitudinal sections the anterior end of the egg

is seen to have a denser cytoplasm than the rest. This is identified as the polar plasm, containing the cast-off polar body. Very soon a structureless vitelline membrane is formed closely adherent to the layer of cytoplasm beneath it. The cortical layer of cytoplasm so closely follows the contour of the vitelline membrane that it is thicker at the angles than elsewhere.

The outer part of the cortical layer of cytoplasm is more compact (dense) and absorbs stain more readily than the inner half. The cytoplasm inside rapidly loses its homogeneous nature and becomes converted into what may be termed in the broad sense 'yolk' (deutoplasm). Yolk-granules and vacuoles appear in it. The yolk is interlaced by very fine cytoplasmic strands continuous with the cytoplasm of the cortical layer, in the meshes of which the vacuoles are distributed. The nucleus has shifted to the anterior end and is situated on one side of the egg (probably the ventral) very near the periphery.

In the stage described above, therefore, three distinct elements are seen in the egg other than the nuclei. (1) The cytoplasm forming the cortical layer and the interlacing network inside; (2) yolk-granules in the meshes; (3) fat globules, the space occupied by them being vacant, owing to their being dissolved by the various reagents used.

In the next stage (fig. 3, Pl. 25) the vitelline membrane is very clearly seen to be in continuity with the cortical layer, which is very thin owing to the yolk-granules taking up all available space. At this stage there appears to be a centrifugal flowing of the cytoplasm which leaves the centre of the egg free from any cytoplasmic material. The chorion is secreted round the vitelline membrane and an outer horny capsule has been formed round the chorion. The egg is laid in this condition.

In the early stages of development the nucleus lies in the centre of the egg, but later migrates towards the anterior end.

Even though a very large number of eggs were sectioned soon after oviposition, the process of cleavage was not observed. In an egg about 10 days old the blastoderm is completed and the embryo formed. Cells are seen to be budded off from the middle of the germ-band (fig. 5, Pl. 25).

The blastoderm is a very thin layer of flattened cells. The nuclei are arranged in groups and placed wide apart.

### III. CHANGES IN THE EXTERNAL FORM OF THE EMBRYO.

The embryo is formed from a specialized part of the blastoderm, termed the germ-band. When first differentiated from the rest of the blastoderm (fig. 7 a, Pl. 25) it is heart-shaped. The changes in shape undergone are: increase in length, decrease in width in the posterior region, increase in thickness, and flexure of the posterior end. The flexure of the posterior end of the germ-band disappears and the embryo straightens itself, the anterior end drawing the rest of the germ-band anteriorwards. It is found on the ventral side of the egg, its anterior end almost reaching the anterior pole of the egg. Later on the posterior end of the germ-band becomes flexed towards the ventral side.

The changes in shape undergone by the embryo of *Carausius* are the same as have been observed in *Blatta* by Wheeler (1889) and are comparable to the shifting of the embryo through the yolk in *Xiphidium* (Wheeler, 1893).

### IV. FORMATION OF THE MESODERM.

Examination of sections of a large number of eggs in which the blastoderm is just formed fails to reveal any trace of primary yolk-cells or their nuclei. The central yolk is one homogeneous mass and does not show any division except at the periphery. A very thin layer of cytoplasm surrounds the yolk. The absence of a trace even of nuclei in the yolk at this stage is ample evidence that the peripheral migration of the products of division of the original cleavage nucleus is complete. Heymons (1897) found primary yolk nuclei in all Orthopterans except in *Grylotalpa*, but Hammerschmidt (1910) and Leuzinger (1926), as in the present investigation, found no primary yolk-cells in *Carausius*. Wheeler (1889) observed in *Blatta* all the derivatives of the cleavage nucleus migrating to the surface to form the blastoderm, but states that at the same time certain cells migrate back to the yolk from the blastoderm.

Closely following the completion of the blastoderm the germ-band appears on the ventral side of the egg near the posterior



pole. Its cells are arranged as a regular columnar epithelium. This is brought about by a contraction of the cytoplasm with its contained nuclei from the dorsal and lateral sides of the egg, as well as from the anterior end of the ventral side. The cells at the anterior end of the germ-band spread sideways forming the rudiments of the cephalic lobes; the unaffected median part forms the anterior ventral groove (fig. 7 *b*, Pl. 25). Elsewhere on the germ-band there is no trace of any groove or invagination. It should be emphasized that in this stage also the yolk-nuclei are absent.

Very soon cells are given off from the middle of the germ-band starting from just behind the anterior ventral groove. This proliferation continues to the caudal end of the embryo and the cells migrate into the yolk singly as loose elements (fig. 4, Pl. 25). Only the most anterior and posterior ends of the germ-band do not undergo this loss of cells.

These cells fuse to form a membrane between the germ-band and the yolk. This has been termed by Hammerschmidt (1910) the 'Dotterzellenlamella' (figs. 6, 8, Pl. 25). Some of these cells wander farther into the yolk. These yolk-cells are the largest cells found in the embryo, and they take no part in the formation of the mesoderm. They are the primary endoderm cells in *Carausius*, and represent the evanescent endoderm of *Pieris* (Eastham, 1927) and *Calandra* (Mansour, 1927). The significance of this proliferation is far-reaching (see section on the yolk-nuclei).

The formation of the anterior ventral groove has already been described. The middle part of the ventral plate posterior to the cephalic lobes shows no groove. This part is thinner than the lateral sides of the germ-band owing to the movement of endodermal cells into the yolk.

As soon, however, as this thinning is effected the middle part of the germ-band becomes slightly unfolded throughout its length, so that in section it becomes concave. This infolding proceeds from before backwards and is continuous with the anterior ventral groove (figs. 7 *b*, 7 *c*, Pl. 25). The infolding is broader and shallower at the cephalic end. This infolding I regard as homologous to the gastrular furrow. Neither

Leuzinger (1926) nor Hammerschmidt (1910) has observed it, but it can be recognized in Leuzinger's figures.

The mesodermal cells are proliferated from the middle part of the ventral plate, i.e. from the apex of the arch of the gastrular furrow from very near the anterior tip of the germ-band to almost the posterior extremity (fig. 6, Pl. 25). The cells are given off singly or in batches of two and three and the process takes place from before backwards. These cells slide over one another in a transverse direction and occupy the space between the ventral plate and the yolk-cell membrane (fig. 17, Pl. 26). They very soon join together and form a layer one cell thick. Active division of these cells takes place and this results in a multi-layered band of tissue. This is the mesoderm. When this proliferation of mesoderm is completed, the ectoderm is formed from the rest of the ventral plate. In Calandra (Mansour, 1927) the mesodermal cells remain unconnected with each other for a very long time.

From the above description it is seen that in *Carausius* the formation of the mesoderm is effected by a process of cell migration which takes place from cells forming the bottom of the gastrular furrow (or apex of the arch, according to the way in which one looks at it).

In Pterygote insects invagination must have been the fundamental method of lower layer formation, and the gastrular furrow seems to represent a vestige of the process of invagination seen in some other insects.

The following instances support this contention very clearly:

**Orthoptera.**—Bruce (1887) finds a gastrular groove in Mantidae and Graber (1888) in Acridiidae, where cells are budded off along the entire length of the germ-band. 'In all families of Orthoptera examined, except in Phasmodae, the gastrula is invaginate' (Wheeler, 1893). But, as we have seen, a gastrular furrow is found in the Phasmod, *Carausius*, from which the mesoderm arises.

**Odonata.**—Tschuproff (1903) finds in Libellulids mesoderm arising from a longitudinal groove.

**Hemiptera.**—Will (1888) finds a longitudinal gastrular furrow from the sides of which the mesoderm is formed by immigration.

**Trichoptera.**—In Phryganids, Patten (1884) finds a gastrular furrow which 'when closing from behind forwards' encloses a few cells between the yolk- and the germ-band which are the rudiments of the mesoderm.

**Lepidoptera.**—Eastham (1927) finds proliferation and invagination before the overgrowth of the middle plate by the lateral plates of ectoderm. Brobretsky (1878) describes proliferation of mesoderm cells from an invaginated groove in *Porthesia*. The proliferation of cells observed by Eastham (1927) in *Pieris* does not, however, as he supposes, correspond to the proliferation of mesoderm in Orthoptera, but is really the rudiment of the endoderm.

**Coleoptera.**—Longitudinal invagination of the median part of the germ-band gives rise to a groove or tube from the walls of which mesoderm cells are proliferated. (It is a groove in *Calandra*, Mansour, 1927.)

**Diptera.**—Kowaleksky (1886) found in *Musca* an invagination of the middle part of the germ-band from the walls of which the mesoderm was formed.

**Hymenoptera.**—In *Chalicodoma* (Carrierre, 1890) gastrulation is by invagination. In the drone bee a rolling up of the edges of the middle plate is observed, but an invagination was not found in the worker eggs of *Apis* (Nelson, 1915).

In the representatives of the various insect orders cited above, whatever be the final method of germ-layer formation it is preceded by some invagination, and therefore it can be concluded that the fundamental method of gastrulation in insects is by invagination, as suggested by Wheeler (1898), and not by proliferation of cells, as stated by Heymons (1895).

Though the lower layer is formed by immigration of cells singly or in batches, these early become aggregated into small groups of cells, which are then arranged in segmental masses. In the anterior region of the germ-band the segmental masses are separated from each other entirely. Transverse sections through the intersegmental grooves show no trace of mesoderm (fig. 22 b, Pl. 26). Towards the posterior end the segmental masses are not cut off in a like manner: the mesoderm is found on the lateral margins of the intersegmental spaces, only the median

portions of these spaces being devoid of it (figs. 22 *b*, 22 *d*, Pl. 26). The band of mesoderm gets wider as one proceeds backwards, a condition very much to be expected, inasmuch as the farther back one goes the less strongly marked is the segment.

Later changes in the mesoderm are mostly concerned with organogeny. The segmental mesoderm extends laterally, and the median portions of the segmental masses become narrow throughout. At this time increase in the area of the mesoderm is indicated by numerous mitoses so placed that the new cells move towards the sides.

The mesodermal cells divide rapidly within these somites and a small cavity is left in the middle by the cells moving away ventrally and dorsally (fig. 22 *c*, Pl. 26). This is the coelomic cavity of the mesoblastic somite. Only the lateral portions of the mesoderm form the somites, the median part remaining undifferentiated. 'The undifferentiated median part of the mesoderm is formed by the precocious breaking down of the somites' (Heymons, 1895).

The middle of the ectoderm now exhibits a deep groove which nearly separates the ectoderm as well as the mesoderm into two parts (figs. 22 *c*, 22 *d*, Pl. 26). This is the neural groove. Large cells are differentiated from the ectoderm on either side of this groove. These are the neuroblast cells, from which the nerve-chain originates. Tracheal invaginations can now be seen in the segments in which stigmata occur in the adult. At this stage these invaginations are ventrolateral in position.

#### V. FORMATION OF THE ENDODERM.

There is no subject in insect embryology which has received more attention than the formation of the endoderm (the rudiments of the mid-gut epithelium). The interpretations and conclusions are so varied that it is impossible to harmonize the results. It is hard to say whether these differences are the results of errors in observation or interpretation, or whether the developmental processes differ widely in closely allied orders of insects. During the last fifty years this problem has been the subject of study of numerous investigators, and their views have

been discussed by Korschalt and Heider (1899), Nelson (1915), Eastham (1930), Henson (1931), and others.

Hammerschmidt (1910) found that in *Carausius* the splanchnic layer of the mesoderm gave rise to the lining of the mid-gut. The cells proliferated from the middle of the ventral plate form the yolk-cell lamella. This is endodermal in nature and transitory. Strindberg (1914) in the same material found a splanchnic mesodermal origin for the mid-gut epithelium. He found that the cells passed inwards are endo-mesoderm, the endoderm being formed by delamination along the entire length of the germ-band. In 1926 Leuzinger, Weismann, and Lehmann identified the yolk-cell lamella of *Carausius* as the true endoderm. The lining of the gut is, according to them, formed by proliferation of cells from the median margins of the segmentally arranged coelomic sacs as well as from the blind ends of the stomodaeum and proctodaeum. These cells gradually replaced the endodermal cells of the yolk-cell membrane. They maintain that in the post-embryonic stages of the stick-insect the true endoderm is absent and that the adult mid-gut is ectodermal.

**Descriptive.**—Two rudiments which appear as heaps of cells and arise at the anterior and posterior ends of the germ-band in the regions of the future mouth and anus give rise to the epithelium of the mid-gut in *Carausius*. These rudiments are endodermal in nature.

**The Anterior Endoderm Rudiment.**—It has been pointed out that the anterior extremity of the germ-band fails to keep pace in growth with the lateral edges when the cephalic lobes are formed, thus giving rise to the Anterior ventral groove. This part of the germ-band is about  $500\mu$  in width, whereas the rest is only about  $250\mu$ – $300\mu$ . The gastrular furrow is formed posteriorly to the cephalic lobes. The stomodaeum has not as yet appeared.

This is the place where the anterior endoderm rudiment is formed. It manifests itself as a rapid proliferation of cells which results in the formation of a rounded mass or heap in the middle of the cephalic lobe (fig. 8, Pl. 25). The regular columnar nature of the ventral plate is disturbed and cells are passed towards its inner side. The area of this proliferation is about  $150\mu$  in

diameter. The cells spread in all directions between the ventral plate and the yolk-cell membrane. The latter is very thin and covers the yolk in the region of the ventral plate. The rudiment pushes against this membrane and the yolk behind it.

In transverse and sagittal sections, a central region is found where the epithelium of the ventral plate is confluent with the newly formed endoderm. Cells in this region are seen to migrate into this mass from the ventral plate (fig. 9, Pl. 25). The line of demarcation between the plate-cells and the endoderm is not precise and never becomes so. The area of proliferation is limited. The endodermal part is easily distinguished by the shape and size of the contained nuclei and the relative amount of stain taken. The endodermal nuclei are rounder and smaller, as a rule, than the elongated and columnar nuclei of the plate-cells. The peripheral cells of the anterior rudiment are sharply defined from the ventral plate-cells over which they have spread. These peripheral cells have been shown to be partly mesodermal in nature (Eastham, 1927; Henson, 1932).

It is seen in sagittal sections that the anterior end of the mesoderm, when it is differentiated from the ventral plate, is in continuity with the posterior end of the endodermal rudiment (fig. 11, Pl. 25). Here we have the ectoderm, endoderm, and mesoderm running indistinguishably into one another, a condition similar to that which was observed by Sedgwick (1885) at the proliferating oral blastoporic area of *Peripatus*.

Soon after the formation of the endoderm rudiment an invagination of the ventral plate beneath the proliferating area occurs which pushes the rudiment still farther inwards into the yolk. This is the stomodaeum (figs. 10, 11, Pl. 25). The proliferating area diminishes in size. The endoderm is then found as two masses on the ventro-lateral sides of the still elongating stomodaeum just posterior to its tip (fig. 12, Pl. 25).

**The Posterior Endoderm Rudiment.**—The development of the posterior endoderm rudiment differs in no essentials from its anterior counterpart. The inner margin of the posterior end of the germ-band gives rise to the posterior rudiment (fig. 13, Pl. 26). This develops very much later than the anterior rudiment and appears to be a process continuous in time and

space with the proliferation of the primary endoderm cells. The rudiment can be distinguished from the proliferation along the middle line by the fact that its constituent cells do not migrate towards the yolk, but are all aggregated together and form a heap of cells like the anterior rudiment. The anterior end of this endoderm rudiment is continuous with the posterior end of the mesoderm and is placed in the last abdominal segment (fig. 14, Pl. 26). Very soon the tip of the embryo bends and buries its tail in the yolk. The rudiment also shifts its position (fig. 20, Pl. 26).

As soon as the posterior rudiment has begun to differentiate (fig. 15, Pl. 26) the proctodaeum starts as an inpushing of the ectoderm impinging against this mass. Owing to the very rapid development of the proctodaeal invagination the proliferating mass of endoderm is carried forward on the tip of the proctodaeum far from the place of its origin. Finally it is found as two small masses of cells placed ventrally very near the blind end of the proctodaeum (figs. 16, 19, Pl. 26).

The anterior and posterior rudiments thus produced later give rise to the definitive lining of the mid-gut by rapid division and spreading of cells.

Observations in *Carausius morosus* on the formation of the endodermal rudiments of the mid-gut thus agree very closely with the results of Eastham (1927, 1930) for *Pieris rapae*, Henson (1932) for *Pieris brassicae*, Nelson (1915) in *Apis*, Wheeler (1889, 1893) in *Blatta*, &c.; Hirschler (1909, 1912) in *Catocala*, &c. Nusbaum and Fulinski (1909) in *Phyllodromia* and *Periplaneta*. But the results of Heymons (1894, 1895) for Orthoptera, Graber (1891), Korotneff (1891), Mansour (1927) in *Calandra*, Hammerschmidt (1910), Strindberg (1914), and Leuzinger, Weismann, and Lehmann (1926) in *Carausius* itself are contrary to the above observations.

It is interesting to note that most of the recent investigators on the development of the mid-gut rudiments in insects are divided into two camps, viz., those advocating an ectodermal origin from the blind ends of the stomodaeum and proctodaeum, and those advocating an endodermal origin from two

proliferating areas of the blastoderm either dependent or independent of the lower layer.

Eastham (1927, 1930) found in *Pieris rapae* anterior and posterior endoderm rudiments which take part in the formation of the mid-gut. Eastham and Henson (1932) found evanescent endoderm along the middle line of the ventral plate. The latter compared these cells to the cells which form the lips of the long slit-like blastopore of *Peripatus* (Sedgwick, 1884), and the anterior and posterior proliferating centres of blastoderm to the remnants of this blastopore which form mouth and anus. Mansour (1927) found in *Calandra* a median wave of cell proliferation which he regarded as evanescent endoderm. This was the condition for which Wheeler (1893) was searching, viz., endoderm formation along the whole embryo, the anterior and posterior portions alone persisting, while the median part degenerates after exercising its embryonic function. Kowalewsky (1886) compared the insects to *Sagitta* as far as the manner of gastrulation was concerned, and supposed that the endoderm was developed from end to end continuously between the lateral invaginations of the mesoderm. In insects, owing to the great elongation of the gastrula, the endoderm was suppressed except at the two extremities. This view is greatly strengthened by Wheeler's (1893) observations in *Stagmonantis*, where the gastrular groove is so short that the oral and anal endodermal centres are almost confluent. Separation of endoderm into two pieces is easily explained by the rapid elongation of the ectoderm and mesoderm with which the endoderm does not keep pace. When the embryo shortens again it obviates the necessity for the third layer also to be shortened to accommodate itself in the space available on the yolk surface.

Fernando (1934) found in *Archipsocus Fernandi* anterior and posterior endoderm masses giving rise to a middle mass of endoderm cells which form a lining on the ventral side of the nutritive mass. This is supplemented by further immigration of cells from the two rudiments. These cells form the definitive lining of the mid-gut. The anterior and posterior masses themselves form evanescent endoderm vesicles which are digested by the gut after the completion of the latter.



Nelson's (1915) conclusions on the honey-bee are very interesting, since the final result that could be arrived at depends on the interpretation. First, the mesenteron may be regarded as the mesoderm, owing to the fundamental similarity of origin of the two organs, but on the other hand the subsequent fate of the endoderm is very different from that of the mesoderm.

As against these views Heymons (1895) and his supporters maintain that the adult mid-gut of insects is ectodermal. According to them true endoderm does not enter into the post-embryonic stages of insects. In the recent work of Mansour (1927) on *Calandra* and of Leuzinger (1926) on *Carausius morosus* both these authors support this view. In both insects the authors assert that the mid-gut arises from tissue other than endodermal. Eastham (1927, 1930) suggests that there may not be very great distinction between a mid-gut which develops from anterior and posterior endoderm rudiments and one which arises by proliferation from the blind ends of the stomodaeum and proctodaeum, and hence is interpreted as ectodermal. It is difficult to visualize how there can be so great a difference in such a vital matter as the development of the mid-gut in a group of animals which exhibit so close a uniformity of structure.

We can imagine the germ-band of insects to consist of several parts and their cells to be indistinguishable from each other in the early stages. On the surface is the material from which the ectoderm will arise (i.e. the primordium of the ectoderm), and within this there are three longitudinal bands, the median one, enlarged at either end, forming the middle strand from which the endoderm rudiments develop, and two lateral bands representing the mesoderm rudiments (fig. 18, Pl. 26).

In some insects, at the time of gastrulation, the endodermal and mesodermal strips including the extremities of the endoderm (mid-gut rudiments) are pushed inwards. Here the mid-gut may be formed from the two ends of the endoderm only (e.g. *Apis*, *Gryllotalpa*, *Blatta*, *Pieris*, &c., and *Carausius*) or the middle strand also may take part in its formation (e.g. *Periplaneta*, *Phyllodromia*, *Catocala*, &c.).

On the other hand, at the time of gastrulation the terminal

parts of the endoderm may be retarded in development and left behind till they are carried inwards on the tip of the stomodaeal and proctodaeal invaginations. Here we get a condition producing the appearance of an ectodermal proliferation similar to that observed by Mansour (1927) in *Calandra*. In other words, in those insects which do not show an obvious endodermal proliferation the endoderm is retarded in development, and hence the tips of the stomodaeum and proctodaeum of such insects are not purely ectodermal but carry endodermal elements also. The difference between the two types of development as typified by *Carausius* and *Calandra* would therefore only concern the relative times at which the mesenteron rudiments are formed by proliferation and the stomodaeum and proctodaeum formed by invagination of the ectoderm.

In 1909 Nusbaum and Fulinski suggested that, in insects 'hitherto regarded as having an ectodermal mid-gut, either the endoderm rudiments were formed late, or the stomodaeum and proctodaeum developed early, so that the endoderm appears to have an ectodermal origin in the stomodaeum and proctodaeum' (quoted from Eastham's 'Review', 1930). They show in support of this that there are seven types of development of the mid-gut rudiment depending on the precocity or lateness in the appearance of the stomodaeum and proctodaeum compared with the mid-gut. This view is fully supported by the conclusions of the present author, produces uniformity of interpretation where none existed before, and brings into line all the different theories on the development of the mid-gut rudiments.

## VI. THE YOLK AND YOLK-NUCLEI.

When the blastoderm is completed the yolk presents a peculiar structure. The central part is a homogeneous mass and no cleavage of the yolk is seen. But the surface-layer of the yolk is cut up into numerous yolk-globules with large spaces in between them. It has already been mentioned that at this time the deep-lying as well as the peripheral masses of yolk are devoid of any yolk-nuclei.

A secondary cleavage of the yolk has been described for many Orthoptera and Dermaptera (Heymons, 1895). Wheeler (1889)

found in *Blatta* that the segmentation of the yolk took place only very late. In higher orders of insects the cleavage takes place very early and proceeds from the anterior end of the egg backwards (Eastham, 1927). In these the yolk-nuclei are distributed in the yolk-segments. These are derived from the cleavage nucleus and are called the 'primary yolk-nuclei'. In *Carausius* the yolk does not show any cleavage and the primary yolk-nuclei are absent; for, as we have already noted, the entire products of the cleavage nucleus migrate to the surface to form the blastoderm (Wheeler, 1889; Patten, 1884; Korotneff, 1885; Hammerschmidt, 1910; and Leuzinger, 1926).

Even in those insects where the yolk-nuclei are present, observations on the fate of these nuclei are few and inconclusive, primarily because the early embryologists believed them to be the definite endoderm which gave rise to the mid-gut; and then, when another mode of origin had been assigned for the latter, the interest in the yolk-nuclei waned.

Soon after the germ-band is differentiated from the blastoderm a tangential proliferation of cells is observed (fig. 4, Pl. 25) from the middle of the germ-band. These cells, as already mentioned, form the yolk-cell membrane, which was supposed by Hammerschmidt (1910) and Leuzinger (1926) to give rise to the lining of the mid-gut. Some of these cells wander farther inwards into the yolk, retaining a connexion with the membrane by cytoplasmic strands. These latter cells are smaller in size and of a distinctly different shape from those forming the membrane.

The yolk-cells increase in size very rapidly and become the largest cells in the yolk at this time. Their outline is irregular, and one or two nucleoli are found inside them. More commonly the chromatin granules are scattered (fig. 5, Pl. 25). The cells were never observed to divide. These cells take no part in the formation of the germ-layers. They disintegrate separately in the yolk, and cells in all stages of disintegration are found. The granular nature of the cytoplasm is lost, vacuoles appear, the cells shrivel up and take stain less readily. Finally they are found as weakly stained circular patches enclosing dark-stained crescentic bodies (fig. 5, Pl. 25), the degenerated remains of

nuclear material. The yolk-nuclei probably function in the early embryonic stages by liquefying the yolk, and rendering it capable of being absorbed by the embryo.

These cells are therefore endodermal in nature and evanescent in character. These are the only yolk-cells found in *Carausius*.

Graber (1871, 1878) was the first to observe the immigration of cells from the germ-band into the yolk, and Heymons (1895) has given a complete account of this process. Korotneff (1885, in *Gryllotalpa*) and Wheeler (1889, in *Blatta*) observed this phenomenon. Heymons paid special attention to these cells in the Orthoptera and called them 'Paracytes'. He distinguished them from the other embryonic cells by the dissolution of the nucleus and the very characteristic separation of the chromatin. The same has been found in other groups of insects also (e.g. in *Musca*, wall-bee, honey-bee, *Pieris*, &c.). The observations of Hammerschmidt (1910), Strindberg (1914), and Leuzinger (1926) on these cells are similar to mine.

The presence of the yolk-cell membrane in the stick-insect closely recalls the condition found in Hymenoptera (Nelson, 1915), and that found by Mme Tschuproff-Heymons (1899) in the Odonata. This membrane might represent the vestiges of an ancient mid-gut epithelium which was primitively formed from the yolk-cells. In the Psocoptera (Fernando, 1934), endodermal cells in the nutritive mass which correspond to the yolk-cells of other insects take part in the mid-gut formation.

In the Orthoptera the primitive method of mid-gut formation might have been by proliferation from the gastrular groove along the entire embryo, the cells in the middle taking an active part. This condition is still found in *Periplaneta*, *Phyllo-dromia*, &c. Later, when the yolk-nuclei separated off from the middle region of the blastoderm earlier than at the extremities, they tried to form the mid-gut by themselves, but in their attempt they reached no farther than the embryonic stages, where they function as vitellophags. The yolk-cell membrane represents this stage of development. When the mid-gut originates from the two endoderm rudiments, the primary endoderm (i.e. the yolk-cells) disappears. It is worth noting that

something very similar to this occurs in the yolky eggs of the Cephalopoda. Here also a yolk-membrane is formed, but the mid-gut arises independently of it from a group of cells which also give rise to the mesoderm.

#### VII. THE DEVELOPMENT OF THE GUT.

The alimentary canal develops in three sections, the fore-, mid-, and hind-gut. The mid-gut is of the greatest interest because of its peculiarities of development. As has already been pointed out, an examination of the literature on this subject brings out with great force that lack of agreement depends more on differences in interpretation than on differences in the actual mode of development.

**The Fore-gut.**—The stomodaeum starts development as a very shallow invagination in the centre of the anterior part of the germ-band, impinging against the proliferation of the endoderm rudiment (fig. 10, Pl. 25). This is placed at right angles to the surface; then, as the prae-oral ectoderm grows, it gets bent backwards. It grows rapidly and becomes in later stages a simple tube folded once or twice. No valves or folds are formed. The tip of the stomodaeum pushes against the endodermal cells and carries parts of the latter inwards. This tip changes in shape; the floor of the stomodaeum becomes thin and gradually widens out so that it assumes a club-shaped form (fig. 12, Pl. 25). Sections show that its dorsal and ventral walls are unequally thickened and that the tip is extremely thin. The stomodaeum is surrounded by the mesoderm of the head segments, the ventral side being comparatively free. In this region the sub-oesophageal body is found.

**The Hind-gut.**—The proctodaeum is very similar to the stomodaeum but it is very much delayed in development. Its place of origin is in the last abdominal segment a little in front of the posterior endoderm rudiment (fig. 15, Pl. 25). The invagination is directed anteriorly. The proctodaeum develops so rapidly that it carries the proliferating mass of endoderm along with it at its tip. It very soon reaches the same stage of development as the stomodaeum. The proctodaeum is invested with a uniform covering of mesodermal tissue. This is the

mesoderm of the last abdominal segment which was carried away from its place of origin by the growing proctodaeum. In the adult, the last abdominal segment is almost devoid of mesoderm.

The Mid-gut.—The anterior and posterior mid-gut rudiments differ from the mesoderm in their mode of formation and their nuclei are distinct. The time of development of the anterior and posterior masses differs, the former marking the beginning, and the latter the close, of gastrulation process. Consequently, at the close of gastrulation, the anterior rudiment is much larger than the posterior one. When the stomodaeum and proctodaeum appear, proliferation has almost ceased at the anterior end, while it has only just started at the posterior end, so that the posterior rudiment on superficial observation appears to develop from the ectoderm of the proctodaeum. The anterior rudiment spreads backwards, its two lateral edges moving very rapidly between the yolk and the mesodermal somites. These are in the form of two tongues of cells (fig. 12, Pl. 25), and become closely applied to the splanchnic mesoderm. Cell divisions take place in a direction transverse to the plane of growth. The closeness of the mid-gut cells to the mesoderm gives rise to the erroneous impression that the former is delaminated from the latter. The development of the posterior strands of endoderm in no way differs from the anterior ones (fig. 16, Pl. 26). From the two pairs of endodermal strands thus produced the entire mid-gut is formed. The anterior and posterior bands meet each other about the third abdominal segment. By rapid cell division they spread between the mesoderm and the yolk in the form of a plate, later becoming a gutter-shaped groove. The lateral edges of this groove grow dorsalwards and form a tube surrounding the yolk. The floors of the stomodaeum and proctodaeum form lamellae dividing the cavity of the mid-gut from the outside (fig. 19, Pl. 26).

#### VIII. THE MALPIGHIAN TUBULES.

The partition separating the cavities of the mid-gut and hind-gut is not broken down till very near the time of hatching. Henson (1932) asserts that in the Lepidoptera examined by him this partition is endodermal in nature (v. his Text-fig. 8), but the examination of sections of *Carausius* embryos at various

stages of development makes it clear that the endoderm of the mid-gut does not extend into this partition which is formed exclusively from the ectodermal lining of the hind-gut. This lining is prolonged forward along the sides of the mid-gut as two plates of ectoderm. On these plates the rudimentary Malpighian tubules open, and, as the cells of these tubules are identical with those forming the plates, it is clear that the tubules are of ectodermal origin (fig. 19, Pl. 26). Statements that the tubules are of endodermal origin appear to be based on the circumstance that these ectodermal plates flanking the mid-gut have been overlooked.

The Sub-oesophageal Body is found as a row or two of very large vacuolated cells ventral to the blind end of the stomodaeum (fig. 12, Pl. 25), which stain only very faintly. Their development was not followed sufficiently to warrant a positive statement about their origin; but from the evidence on hand they appear to be mesodermal. Some authors have asserted an endodermal origin for these cells. They may represent the mesoderm of the pre-mandibular segment (Wheeler, 1893; Eastham, 1930).

#### IX. AMNION AND SEROSA.

The cells of the germ-band are columnar and they differ from the cells of the rest of the blastoderm. The latter are squamous, roughly polygonal, and very widely distributed. This extra-embryonic blastoderm forms the serosa (fig. 7 *d*, Pl. 25). It is in contact with the embryonic rudiment on all sides.

The junction of the serosa and the germ-band is the seat of very rapid cell proliferation. This proliferation begins at the anterior end. As a result the edges of the embryo gradually sink into the yolk and the serosa grows backwards as a sheath. The sunk-in part of the rudiment still retains its connexion with the growing serosa by a layer of cells which constitutes the rudiment of the amnion (fig. 11, Pl. 25, fig. 13, Pl. 26). These cells are rounder than the cells of the embryo and the serosa, and are differentiated from the edge of the embryonic rudiment. This connexion also is made at the anterior end of the germ-band. The serosa and the amnion grow backwards; but the serosa soon overgrows the accompanying amnion, leaving the latter far behind.

By the time the serosa has covered about half the embryonic rudiment the amnion is formed in the posterior end of the germ-band. Eventually the serosa covers the germ-band entirely. The amnion is still rudimentary, being round only along the sides of the embryo. When the embryo undergoes rapid changes in shape the amnion is completed and it is then found as a very thin membrane investing the embryo and enclosing the amniotic cavity between it and the germ-band (fig. 20, Pl. 26).

The amnion when formed is in close contact with the inner side of the serosal epithelium (fig. 21, Pl. 26), but when the embryo sinks down it is usually stated that granules of yolk pass in between the two embryonic coverings and the amnion thus becomes completely separated off from the serosa. The process of separation was not observed in *Carausius*. In the *Lepidoptera* Eastham (1927) shows it to be due to progressive delamination of the one from the other. In the *Orthoptera* investigated by Wheeler (1893) and Heymons (1895) the development of the amnion bears very important relations to the revolutions of the embryo in the yolk. The same is true for *Carausius* also, though not to such a high degree as in the other *Orthoptera*.

#### X. SUMMARY.

1. The maturation of the egg takes place in the ovarian tube, and is immediately followed by the formation of the cleavage-nucleus and its division into many nuclei.
2. The entire products of the cleavage-nucleus migrate to the surface to form the blastoderm. Cleavage of the yolk was not observed even in late stages. Yolk-cells are absent when the blastoderm is being formed.
3. Primitive endodermal cells are proliferated from the middle of the germ-band, and form a membrane between the germ-band and the yolk. The membrane is present only in embryonic stages; some of the cells proliferated wander into the yolk and act as vitellophags.
4. Mesoderm is formed by proliferation of cells from the ventral plate. It is preceded by the formation of a shallow gastrular furrow, and from the bottom of this furrow proliferation



takes place. The mesoderm becomes arranged in segmental masses.

5. Two masses of cells proliferated at the anterior and posterior ends of the germ-band are shown to be the endodermal rudiments from which the mid-gut epithelium is formed. The invaginations of the stomodaeum and proctodaeum grow against these masses and carry parts of the proliferating areas near their blind ends. It is shown that the various methods of mid-gut formation which have been described could be reconciled with the process described in *Carausius*.

6. The hinder end of the mid-gut is flanked by two plates of ectoderm which are forward extensions of the proctodaeum. Into these extensions the Malpighian tubules open, and, as their histology is identical with that of these extensions and widely different from that of the mid-gut, these tubules must be ectodermal in nature.

7. The formation of the amnion and serosa are described.

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## XII. EXPLANATION OF PLATES 25 AND 26.

All figures are Camera lucida drawings. 1/12 oil imm. objective and No. 1 eye-piece, except where otherwise stated.

### PLATE 25.

Fig. 1.—Transverse section of an egg from the ovary at the earliest stage.

Fig. 2.—Longitudinal section of an egg slightly older than above.

Fig. 3.—Transverse section of an egg before the capsule is secreted.

Fig. 4.—Sagittal section of an embryo showing proliferation of yolk-nuclei.

Fig. 5.—Degenerating yolk-nuclei.

Fig. 6.—Sagittal section of an embryo showing the formation of mesodermal cells.

Fig. 7.—Drawings of whole mounts (*a*) embryonic rudiment at its first appearance at the posterior pole of the egg, (*b*) the rudiment elongating, (*c*) the appendages appearing.

Fig. 8.—Sagittal section of the anterior endoderm rudiment.

Fig. 9.—Transverse section of the anterior rudiment in the same stage as in the previous figure.

Fig. 10.—Transverse section, slightly oblique, showing the beginning of the stomodaeum.

Fig. 11.—Sagittal section, same stage as the previous figure.

Fig. 12.—Sagittal section of the stomodaeum showing the anterior endoderm rudiment giving rise to the lining of the mid-gut.

### PLATE 26.

Fig. 13.—Sagittal section of the posterior end of the embryo showing the proliferation of the posterior endoderm rudiments.

Fig. 14.—Sagittal section, older than the above. The posterior mass is very prominent.

Fig. 15.—Sagittal section, showing the proctodaeum as a very shallow depression.

Fig. 16.—Sagittal section showing the posterior endodermal rudiment giving rise to the mid-gut cells.

Fig. 17.—Transverse section showing the mesodermal cells spreading sideways.

Fig. 18.—Hypothetical drawing of the arrangement of the primary germ layers.

Fig. 19.—Longitudinal section of the posterior end of the embryo, showing the ectodermal nature of the Malpighian tubules (1/6 obj.; eyepiece 4).

Fig. 20.—Transverse section of the posterior endoderm mass.

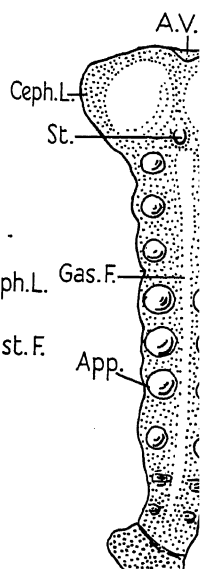
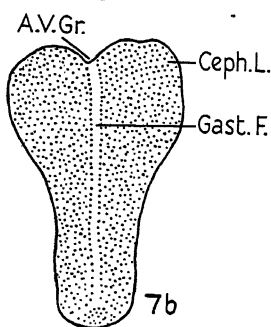
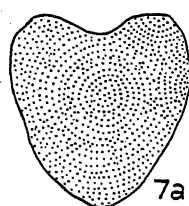
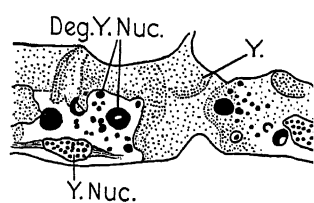
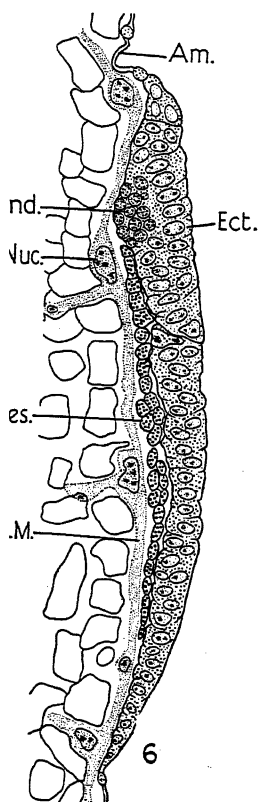
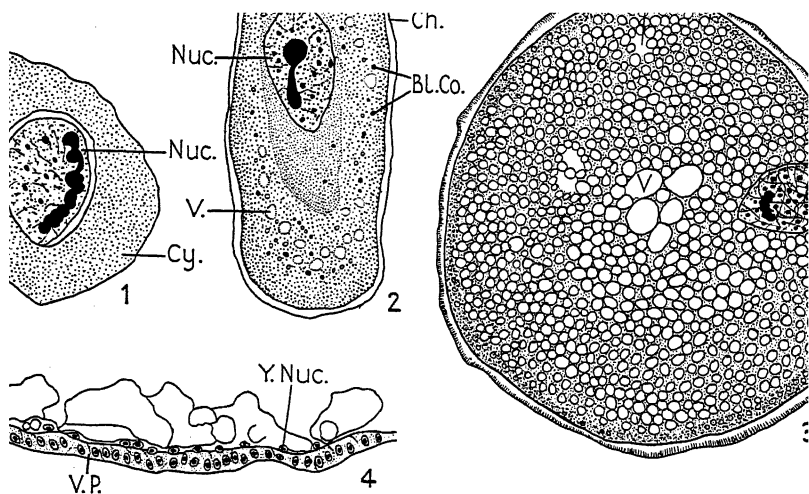
Fig. 21.—Longitudinal section showing the arrangement of the mesoderm.

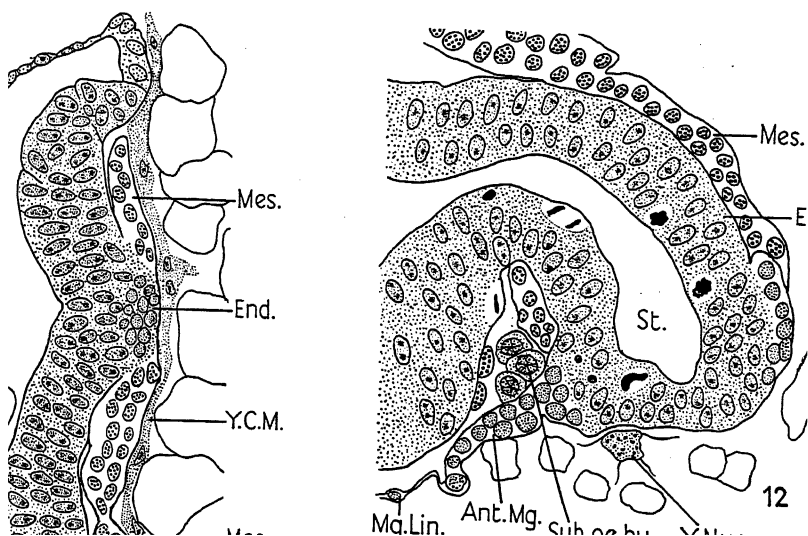
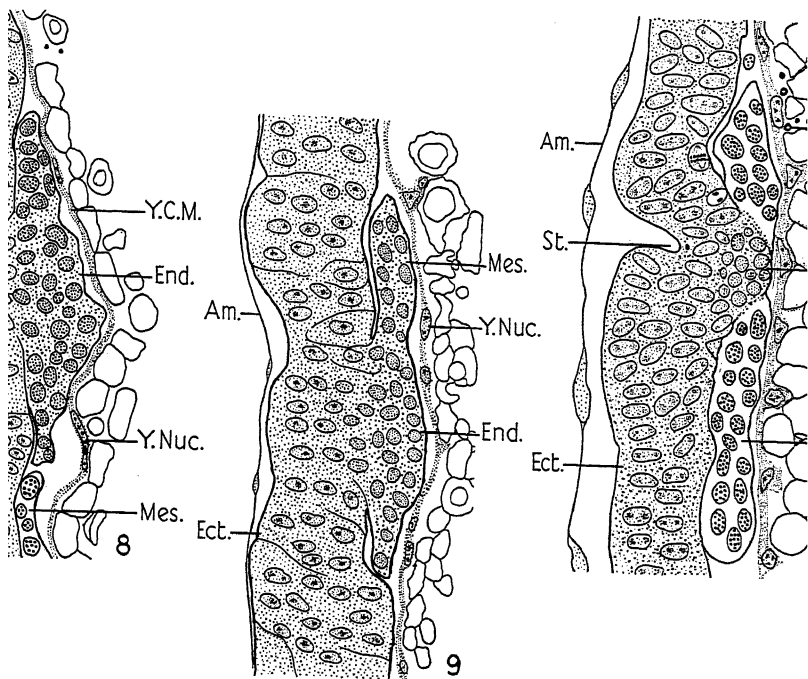
Fig. 22.—Transverse sections showing the arrangement in different parts of the body:

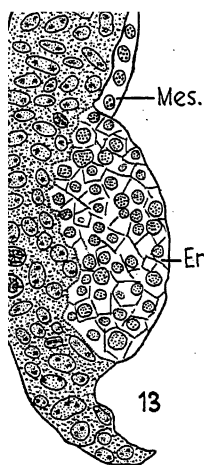
- (a) Segmental mesoderm in anterior region.
- (b) Mesoderm absent in intersegmental region of anterior part. (1/6 obj.; eyepiece 3.)
- (c) Segmental mesoderm and coelomic cavity in posterior region. (1/6 obj.; eyepiece 3.)
- (d) Intersegmental mesoderm in posterior region. (1/6 obj.; eyepiece 3.)

#### ABBREVIATIONS.

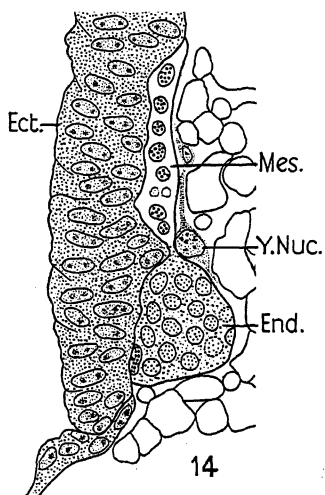
*Am.*, amnion; *Ant.Mg.*, anterior mid-gut rudiment; *A.V.Gr.*, anterior ventral groove; *App.*, appendage rudiment; *Bl.Co.*, Blochmann's corpuscles; *Ceph.L.*, cephalic lobes; *Ch.*, chorion; *Cor.*, cortical layer of cytoplasm; *Deg.Y.Nuc.*, degenerating yolk nucleus; *Ect.*, ectoderm; *End.*, endoderm; *Fl.T.*, flexed tail of embryo; *Gast.F.*, gastrular furrow; *Intseg.mes.*, intersegmental mesoderm; *Mal.T.*, malpighian tubules; *Mal.T.Op.*, opening of the Malpighian tubule; *Mes.*, mesoderm; *Mes.So.*, mesodermal somites; *Mg.*, mid-gut; *Mg.Lin.*, mid-gut epithelium, i.e. the thin, flat cells covering the central part of the mid-gut which are proliferated from the anterior and posterior endoderm rudiments; *M.st.End.*, middle strand of the endoderm; *N.Gr.*, neural groove; *Nu.*, nucleus; *Pos.Mg.*, posterior mid-gut rudiment; *Pp.*, polarplasm; *Pr.*, Proctodaeum; *Seg.mes.*, segmental mesoderm; *Ser.*, serosa; *St.*, stomodaeum; *Sub.oe.by.*, sub-oesophageal body; *V.*, vacuoles; *V.P.*, ventral plate of blastoderm; *Vit.m.*, vitelline membrane; *Y.*, yolk-granules; *Y.C.M.*, yolk-cell membrane; *Y.Nuc.*, yolk nucleus.



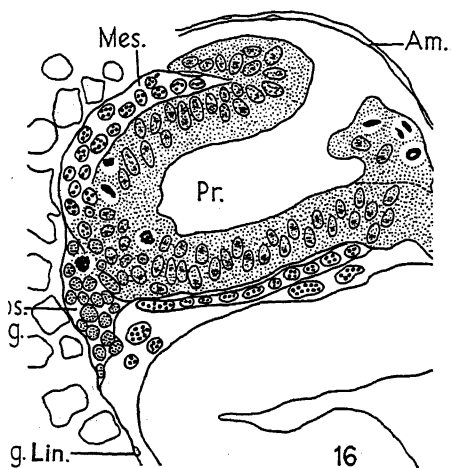
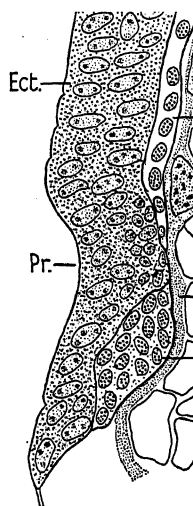




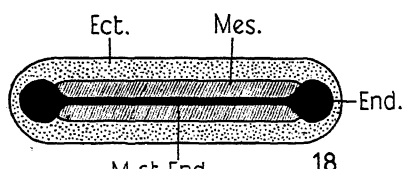
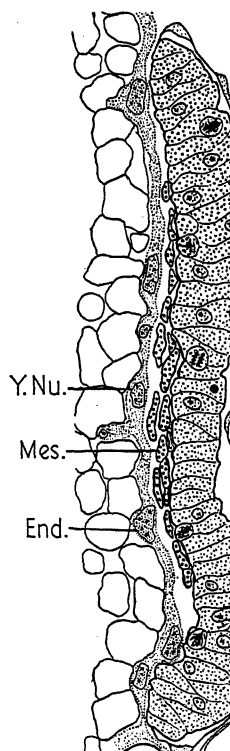
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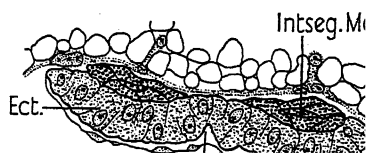
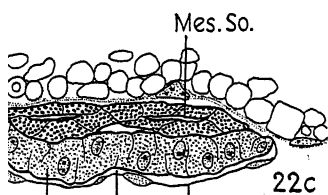
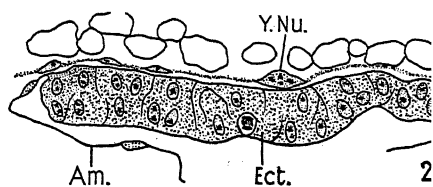
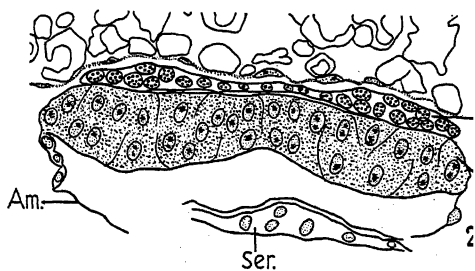
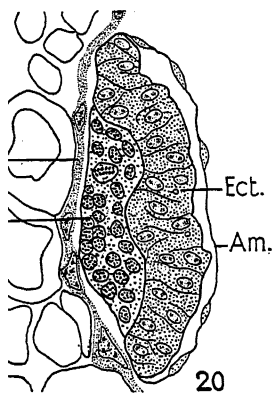
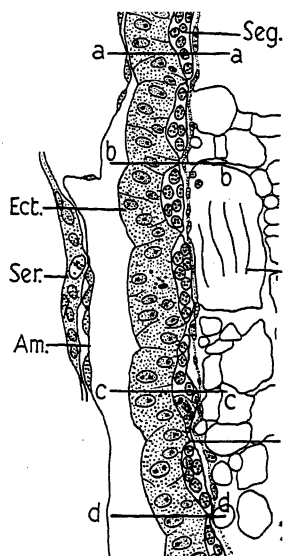
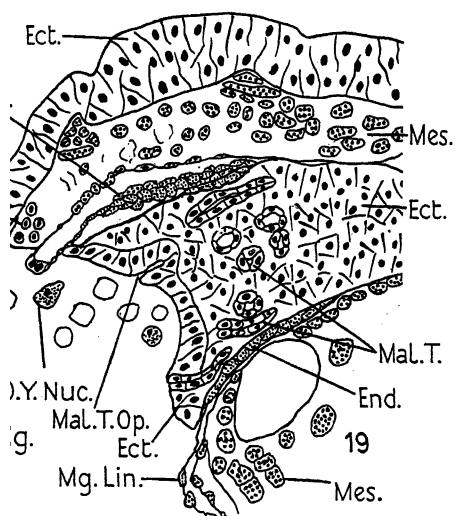
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# **A Study of the Yolk (Y-granules) of the Male Germ-cells.**

By

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With Plate 27 and 2 Text-figures.

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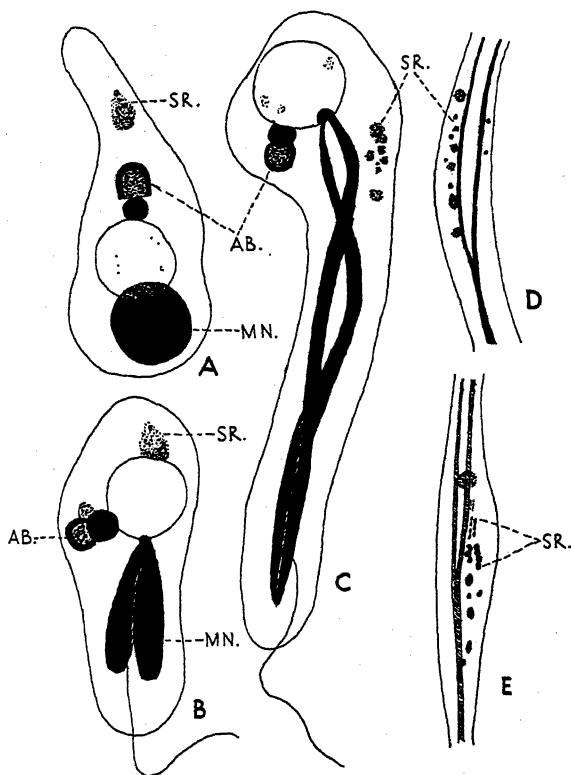
## INTRODUCTION.

It has been established by recent research that the Y-granules exist as a fundamental part of the constitution of the male germ-cells of many animals—vertebrates as well as invertebrates. These granules, wherever present, whether in the germ-cell of a moth like *Abraxas grossulariata*, or in the germ-cell of a mammal like *Cavia cobaya*, exhibit with rare exceptions a remarkable uniformity in their appearance, in their behaviour during the successive stages of the developing cell, and in their reactions to certain fixatives, stains, and vital dyes. This uniformity is so pronounced that it is considered as indicating a similarity in origin, nature, and function of these granules wherever present.

Up to the year 1922, or thereabouts, nothing further was known about these granules beyond what Gatenby had recorded in his study on the gametogenesis of *Saccocirrus* (5). In 1922 he discovered them in the male germ-cells of this archianelid, and gave a description of the behaviour of these granules in the progressive stages of the cell. In none of the other types of spermatogenesis studied did Gatenby find anything approximating in structure or in behaviour to the Y-granules of *Saccocirrus*. Strange as this might appear, it is easily explained by the fact that these granules are very easily destroyed by most of the routine fixatives, and are only under rare and exceptional conditions—as in the case of the above-

mentioned *Saccocirrus*—visible in fixed preparations. So long as fixed material continued to be the exclusive source of information in all cytological investigations, so long did *Saccocirrus* continue to be the one isolated instance in which the Y-granules were known to exist. Reference should, however, be made to the so-called 'spermatid remnant' of Bowen (2), a peculiar cytoplasmic body observed by him in the spermatids of developing spermatozoa of certain pentatomid bugs. Bowen's description of the origin, structure, position, and fate of the spermatid remnant leaves little room for doubting that he is dealing with a cell component very similar to the Y-granules. Text-fig. 1, A–E, illustrates the behaviour of the spermatid remnant in spermatids at different periods of growth. The similarity to Y-granules is very striking. Quite recently Johnson (15), working with Gryllid germ-cells, observed in spermatids fixed in Benda's fluid, bodies similar to Bowen's 'spermatid remnant', Text-fig. 2, A and B, and on comparing them with his 'rubrophile granules' (Y-granules) in vitally stained material (Text-fig. 2, C and D) he expressed the view that judging from its position, structure, and fate, the spermatid remnant is possibly identical with, or derived from, the group of rubrophile granules (Y-granules). The writer would have accepted the view of Johnson but for certain facts. In almost every case where the Y-granules have been studied with the aid of vital staining techniques they were seen in early growing spermatocytes, and one of the most diagnostic characters of the Y-granules is their behaviour during the maturation stages of the cell—particularly during the metaphase and telophase stages. Not only is Bowen silent with regard to the presence of the so-called spermatid remnant in the earlier stages of the cell, but he is emphatic in his view that this remnant makes its appearance only from the spermatid stage onwards. He compares his spermatid remnant with the fat droplets of Duesberg (33) and von Ebner (34). He is also of the opinion that the granules into which this remnant ultimately breaks up are, after ejection from the spermatozoon, engulfed by the epithelial cells of the testis—a phenomenon not yet observed in the case of the Y-granules. Further investigation on pentatomid material with the aid of vital staining techniques would have

to be made before it could be established that the spermatid remnant is identical with the Y-granules. The most that can be said at present is that the spermatid remnant stands in very close relationship to the Y-granules.



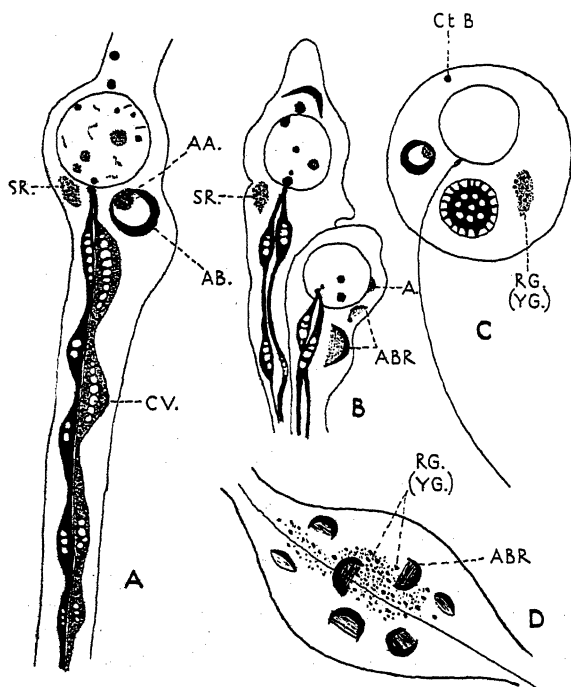
TEXT-FIG. 1.

The 'spermatid remnant' of Pentatomids (after Bowen).

Figs. A-E show the movements of the 'spermatid remnant' during spermatoleosis. Abbreviations: AB., acroblast; MN., mitochondrial nebenkern; SR., spermatid remnant. (Technique: Flemming without acetic-haematoxylin.)

With the application of vital staining techniques to germ-cell problems, it was soon discovered that the Y-granules were not found in *Saccocirrus* only. In 1928 Hirschler (11) demon-

strated the Y-granules in the germ-cells of *Macrothylacia rubi* by vitally staining the cells with neutral red. Soon he supplemented this instance with several others (12, 13, and 14).



TEXT-FIG. 2.

The 'spermatid remnant' and the 'Rubrophila' granules (Y-granules) of Gryllids (after Johnson).

Figs. A and B—Spermatids of *Oecanthus nigricornis*. (Technique: Benda's fixation and staining.)

Figs. C and D—Spermatids of *Oecanthus niveus* and *Oecanthus quadripunctatus* respectively, supravitaly stained with neutral red, showing rubrophile granules (Y-granules). Compare with 'spermatid remnant' in A and B. Abbreviations: AA., acrosome anlage; A., acrosome; AB., acroblast; ABR., acroblast remnant; Ct.B., chromatoid body; RG., rubrophile granules (Y-granules); SR., spermatid remnant.

Gatenby demonstrated them in the moth *Abraxas grossulariata*, in the mammal *Cavia cobaya* (?), and in human

spermatocytes (8) by supravital staining the cells with neutral red. In the years that followed additional instances were produced (15, 23, and 29), so that quite soon after cytologists had seriously taken to the study of vitally stained preparations, the Y-granules were found to exist as a fundamental part of the male germ-cells of several animals representing different orders and families. In a recent communication (24) the present writer has reported the existence of the Y-granules in a number of insects, many of them representing families in which they had not previously been observed. There appears thus to be substantial evidence for considering the Y-granules as a definite male germ-cell constituent; and, as far as insects are concerned, the evidence on record is sufficient to support the view now prevalent that these granules are likely to be of general occurrence. Attention should, however, be drawn to the fact that in none of the Mollusca so far examined have typical Y-granules been observed.

Apart from the bare fact of their existence in the germ-cells of various animals, very little is known as to their origin, nature, or function, and no serious attempt apparently has been made to throw any light on these obscure points. At the suggestion of Dr. Gatenby the writer has recently conducted a series of experiments with the object of ascertaining the origin, nature, and function of the Y-granules. The writer wishes to thank Dr. Gatenby for his many helpful suggestions and criticisms.

#### MATERIAL AND METHOD.

The material used in this investigation was drawn almost entirely from different families of Lepidoptera. It has been found by other workers that the Lepidoptera supplied the best material for the study of the Y-granules. Lepidopterous germ-cells quickly respond to vital staining techniques, and remain healthy for a comparatively long period in smear preparations. It is impossible to exaggerate the value of this point in an investigation of the Y-granules. Of the many types selected, *Abraxas grossulariata* and *Agrotis segetum* proved to be the most satisfactory and, except where otherwise stated, all the observations recorded in this study are from the germ-

cells of these two insects. For a comparative study material was also obtained from *Stenobothrus viridulus* and *Forficula auricularia*.

In the summer of 1934, while examining lepidopterous and other insect germ-cells for Y-granules as a preliminary investigation, the writer often found that in the germ-cells of insects dissected immediately after they had been caught in the field, the Y-granules appeared in a few minutes after supravital staining with neutral red. The reverse was the case in testes taken from insects kept too long in captivity without an adequate supply of fresh food, and the granules were either absent or reduced in number. These preliminary observations were of great assistance in tracing the origin of the Y-granules. It was decided to study more closely the effect of underfeeding and of starvation on the Y-granules. The results of the preliminary tests appeared to suggest that the Y-granules were products of the normal metabolic activity of the cell. The larvae of *Agrotis segetum* were collected and kept in the laboratory in three receptacles marked A, B, and C. In A, the larvae were kept under perfectly clean conditions with a supply of fresh food every 24 hours. In B, the larvae were fed only once in 72 hours, the receptacle being cleaned just when food was supplied. In C, the caterpillars were kept under perfectly hygienic conditions, but without any food. The experiments were repeated with *Abraxa grossulariata*. At regular intervals a certain number of larvae were taken from each of the three receptacles and their germ-cells examined in smear preparations after supravital staining the entire testis with neutral red. After 7 days' starvation the larvae in C were fed regularly, and from the second day of feeding the germ-cells were examined, using the same technique.

For ascertaining the nature of the Y-granules, tests were made with different dyes. Within recent years it has been learnt that the chemical composition of certain cell constituents could be fairly accurately ascertained by studying their micro-chemical reactions to certain fixatives, stains, and vital dyes. As pointed out by Wilson (32), such methods of studying the composition of cell constituents are not wholly trustworthy. Nevertheless,

a fair amount of reliance can be placed on the results obtained by using certain stains and vital dyes.

For determining the composition of the Y-granules the material was studied in fixed, fresh, and supravitaly stained preparations. The Mann Kopsch method as modified by Ludford was the technique adopted in fixing the material. Formalin fixed material post-osmicated, and formalin fixed smears stained with Herxheimer's acetone and scarlet R. (a saturated solution of scarlet R. in equal parts of acetone and 70 per cent. alcohol) and counterstained with methylene blue were also studied. 'Vital' studies were made after supravitaly staining the testis with neutral red, brilliant Cresyl blue, Nile-blue sulphate, and Congo red. As a preliminary trial with Nile-blue sulphate suggested that the granules were of a fatty nature, most of the experiments were directed towards this. To ascertain if there was anything corresponding to the Y-granules in the oogonia and oocytes, the ovaries of *Abraxas grossulariata*, *Gryllus domestica*, and *Lithobius forficatus* were studied after supravitaly staining them with neutral red.

#### OBSERVATIONS.

Effects of normal feeding, underfeeding, and starvation of the Larvae on the Y-granules.

(Controls in every case, larvae collected fresh from the field.)

1. Normal Feeding.—An examination of the germ-cells of normally fed larvae taken from receptacles A and A' (*Agrotis segetum* and *Abraxas grossulariata*) showed practically no difference from the germ-cells of larvae brought fresh from their natural surroundings. The general condition of the cells appeared to be quite normal and the Y-granules in almost every case appeared brightly stained (fig. 1, Pl. 27). The course of the granules could be very clearly followed in all the successive stages of the cell, exhibiting in rare instances certain deviations from the normal behaviour as was observed in *Forficula auricularia* (24), but in every case eventually passing out of the cell without apparently having taken any part in its activities. It was pointed out in a previous communi-



cation (24), that the Y-granules in *Agrotis segetum* distinctly appeared to be situated in a special homogeneous cytoplasmic area or field, quite distinct from the surrounding cytoplasm and which remained unstained, while the granules segregated within it appeared brightly stained.<sup>1</sup>

A careful study of the germ-cells of other lepidopterous types revealed that in almost every case the Y-granules are segregated in a special field. When the field is full of granules its appearance is not very distinct. But in several cells where the granules are fewer in number the area in which they are embedded is clearly visible. Fragments of this area appear to pass with the granules to the daughter cells during the maturation divisions, for even in the advanced stages of the cell it is not uncommon to find two or three granules connected together, as it were, by small strands of a non-staining material which are probably pieces of the homogeneous field. (Figs. 3 and 7, Pl. 27.)

2. Underfeeding.—(Receptacles B and B'.) The examination of the germ-cells commenced 48 hours after feeding. No changes worth mentioning could be observed. An examination made 60 hours after feeding showed no appreciable reduction in the number of granules. An examination made 72 hours after the first feed and immediately before the second revealed a reduction in the number of granules and at the same time the cytoplasm also showed a slight affinity for the stain. An examination of the cells 48 hours after the second feed revealed a normal condition of the Y-granules in the majority of cells, though there were many in which the reduced number was still evident.

3. Starvation.—Data of a very interesting nature could be obtained by examining the germ-cells of larvae kept without food. One of the first noticeable effects of starvation was a shortening of the larval period. There was a marked tendency on the part of many of the larvae to pupate prematurely, and with few exceptions it was therefore only possible to study the effects of starvation on the germ-cells of young larvae. As in the case of those underfed, nothing remarkable was noticed

<sup>1</sup> Hirschler described a similar appearance in *Macrothylacia rubi*, but his idea of the homogeneous field is different from that of the writer.

prior to 60 hours' captivity. The initial reduction in the number of granules was very slight, but after the fourth day the number of granules began to drop steadily and in the germ-cells of larvae examined on the seventh day after captivity very few granules could be seen. In the case of young larvae the cell presented, both in fresh and vitally stained preparations, a somewhat abnormal and distorted appearance.

After 6 days' starvation food was supplied. The majority of larvae showed a disinclination for food, and the examination, 48 hours afterwards, of the few that had taken any food revealed a few granules in some of the healthy cells. In all those cells where disorganization had advanced too far, no improvement was observed. During a repetition of this experiment the larvae were given food after 4 whole days of starvation. From the second day after feeding, a gradual increase in the number of granules could be seen. It should, however, be noted that in all these cases the reduction was more evident in spermatocytes than in spermatids, as was also the increase in the number of granules after feeding. In advanced stages of the cell no appreciable change was noticed after starvation.

Observations on Vitally Stained Cells.—It is common knowledge that the status of the Y-granules as a fundamental part of the constitution of the male germ-cells of several animals came to be recognized only after vital staining techniques with neutral red were used by cytologists. Neutral red has been almost accepted as a specific stain for the Y-granules when supravitaly applied. It is on the strength of their affinity for neutral red that Hirschler called the Y-granules 'rubrophile granula'. But these granules respond as readily to certain other dyes as to neutral red. The writer is decidedly of the opinion that Nile-blue sulphate stains the granules better and quicker than neutral red. An immersion of the entire testis in 0.5 per cent. solution of Nile-blue sulphate stains the granules in five minutes and the staining is most spectacular. In almost every cell the special area of the Y-granules could be seen selectively and intensely stained, while all the other structures remained practically untouched. Whole nests of cells could be seen with just a tiny patch of blue in each cell. The picture is

very convincing. Results very similar to the above were obtained also with brilliant Cresyl blue. The Y-granules were selectively stained a beautiful violet in about ten minutes. The name 'rubrophile granula' therefore appears to be misleading as it suggests that these granules have a specific affinity for neutral red alone, which is not the case.

**Examination of Fixed Preparations.**—Nothing approximating to the Y-granules could be seen in material fixed according to Mann, Kopsch, and Kolatschew techniques. Occasionally in a few cells slightly greyish patches could be seen in the place where the granules usually appear in fresh and vitally stained material. But the writer is not convinced that these are Y-granules.

In smears fixed in formalin vapour and stained with Herxheimer's acetone-scarlet R. solution and counterstained with methylene blue, the Y-granules are the only bodies which appear stained pink (fig. 3, Pl. 27), and this reaction leaves no doubt as to the chemical composition of the Y-granules.

**Examination of ovaries supravitaly stained.**—Fig. 4 in Pl. 27 represents a young oocyte of *Abraaxas grossulariata*. Close to the nucleus occupying almost the same position as that generally taken by the Y-granules in spermatocytes, is an aggregation of neutral red staining granules. Farther removed, a second but smaller aggregation of granules is also visible. At this stage of the oocyte these granules react similarly to Nile-blue sulphate as do the Y-granules in the spermatocytes. As the oocyte grows, the individual granules of the aggregate spread out into the cytoplasm, undergo a process of disintegration, and each of the resulting fragments develops into a sphere. In a fairly well advanced oocyte these spheres are seen scattered indiscriminately in the cytoplasm. When a dilute solution of Nile-blue sulphate is applied to the oocyte some of the spheres appear blue and some reddish. This indicates that the substance of the granules has undergone a chemical change with the increase in size. The fatty acid contents have been probably transformed into fats. A similar phenomenon is observed in the oocytes of *Gryllus domesticus* also. Fig. 5 in Pl. 27 represents a supravitaly stained ovariole of this

insect. In the germarium a few scattered granules are seen stained red, but a juxtannuclear aggregation of neutral red staining granules is visible only in the first well differentiated oocyte in the vitellarium. In an older oocyte the granules are seen wandering out in the cytoplasm and, as in the case of *Abra xas*, they undergo disintegration with subsequent development into fatty spheres. In the case of *Lithobius forficatus*, the disintegration of the primary aggregation of granules and their gradual development into fatty spheres is still more evident. Fig. 9 in Pl. 27 represents a section of the entire ovary supravitaly stained with neutral red. Each of the young oocytes has a juxtannuclear aggregation of red granules surrounded by Golgi dictyosomes. Fig. 8, Pl. 27, shows this aggregate clearly. With the growth of the oocyte (fig. 10, Pl. 27) the aggregation breaks up and the granules slowly wander into the cytoplasm. In the early stages each granule appears to have a dictyosome attached to it. In the later stages the granules, as in the other instances cited, undergo disintegration and increase in size, and many appear free from the dictyosomes. Finally, the fatty spheres are seen scattered uniformly throughout the cytoplasm. Fig. 7, Pl. 27, is a spermatocyte of *Lithobius* showing two aggregates of neutral red staining granules.

#### DISCUSSION.

The results of the experiments conducted for ascertaining the origin of the Y-granules indicate that there exists a definite relation between the Y-granules and the physiological status of the animal. Under normal conditions the Y-granules are always present as could be easily ascertained by the application of any of the vital dyes for which they are known to have any affinity. Likewise their presence could be easily demonstrated in insects kept in captivity, provided that the conditions were not very different from their natural ones. Confinement of large numbers for too long a period in small and ill-ventilated receptacles containing the increasing accumulations of their excreta and discarded food materially affects their physiological condition. When the germ-cells of such undernourished larvae are examined, a reduction in the number of granules is often evident, and

appears to be in proportion to the extent of starvation undergone by the larva. If the food-supply is entirely cut off, even when the insects are kept very cleanly, a steady reduction in the number of granules occurs, until they practically disappear from a number of cells, their absence being more pronounced in spermatocytes than in spermatids. If, after a reasonable period of starvation, feeding is resumed, the granules gradually begin to appear again, except in those cells which appeared to have been adversely affected by long starvation. These facts indicate that the granules are products of normal cell metabolism. Under altered conditions of metabolism induced by undernourishment or starvation the granules steadily disappear. Unfortunately the writer is not aware of any definite attempt that may have been made within recent years to ascertain the effect or effects of underfeeding or starvation on the structure and functional activities of the male germ-cells of invertebrates.

The fact that the germ-cells of heavily parasitized lepidopterous larvae exhibit signs of disorganization, and Y-granules appear only in a few healthy cells, leads the writer to support the view that the Y-granules are products of normal cell metabolism and that their presence or absence depends on the general physiological condition of the animal. The latter state is dependent to a considerable extent on the amount of food available.

**The Composition of the Y-granules.**—Having ascertained that the Y-granules are in all probability metaplastic bodies, their chemical composition has now to be determined. Vital staining techniques as applied in this investigation have been very helpful in the determination of the chemical composition of the Y-granules.

It has been previously noted that the Y-granules can be stained with neutral red, brilliant Cresyl blue, and Nile-blue sulphate. Now all these dyes are known to stain fats and fatty acids, and though neutral red and brilliant Cresyl blue are not specific for fats, in Nile-blue sulphate, as determined by Lorraine Smith (28), we have a very valuable vital dye for the determination of fats and substances closely related to fats. The great advantage Nile-blue sulphate possesses over other stains for fat is that it gives a double, or one might even say

a triple, stain. It colours neutral fat red; cholesterine ester and cholesterine fatty acid mixtures, reddish; sphingomyelin, cerebroside, and kephalin, light bluish; and fatty acids, blue. It is owing to this valuable property that it has come very much to the forefront in recent years as a valuable reagent for testing fats and chemically allied substances. As stated in the section on observations, Y-granules always appeared bright blue when stained with Nile-blue sulphate. According to Lorraine Smith the bright blue reaction indicates the presence of fatty acids in the granules. But Tennent and Gardiner (who made an extensive use of this stain in an analytical study of the ovaries of the sea-urchin, *Echinometra lacunter*), by a series of experiments with known mixtures of triolein and oleic acid (30), ascertained that the blue reaction cannot be regarded necessarily as indicating the absence of neutral fat. One drop of oleic acid added to 20 drops of triolein and 2 c.cm. of water stained all the droplets blue—thus indicating that the trace of fatty acid in neutral fat is sufficient to stain the entire mixture blue. According to this the reaction of Y-granules to Nile-blue sulphate indicates that the granules are either composed wholly of fatty acids or of neutral fat combined with fatty acids. Scarlet R. also stains neutral fat red and fatty acids pink. In coverslip preparations fixed in formalin vapour and stained with Herxheimer's acetone and scarlet R. mixture, the granules were always stained pink. The question now is, are the Y-granules composed wholly of fatty acids or are they a combination of neutral fat and fatty acids? Their reactions to osmic acid indicate that they are almost entirely composed of fatty acids. In fixatives containing osmic acid the Y-granules appeared with a pale grey tinge—almost invisible—an indication of saturated fatty acid. It is probable that the Y-granules are not in every case composed entirely of fatty acids. In certain cases the granules may have a fair percentage of fatty acids. The case of *Saccocirrus*, where the greenish-brown tinge of the Y-granules enabled Gatenby to follow with remarkable precision their behaviour in all the different stages of the cell, indicates that the granules are not always wholly composed of fatty acids. Slight differences in composition are likely to occur not only in

different species, but in the cells of the same species. But one thing is certain, that the granules are composed of a fatty substance and that this substance is elaborated in the course of normal metabolism and segregated in a special area within the cytoplasm. This would account for the granules remaining always as an aggregate, while other structures like mitochondria and Golgi bodies are scattered within the cytoplasm without any special order.

One more question remains to be answered—the question as to the function of the Y-granules. The observations made on oocytes appear to suggest that the Y-granules in spermatocytes are only abortive yolk granules. In the early stages of development these granules are present both in the oocytes and in the spermatocytes. They occupy similar positions in the cells and appear to be substantially the same. But in the oocytes the granules have a specific function. As has been observed, they gradually develop into fatty spheres. In the spermatocytes all observations made so far on normal cells lead to the conclusion that in the natural course of events these granules take no part in any of the activities of the cell. Cytological opinion seems to be unanimous on this point. The Y-granules pass on from one stage of the cell to the other, exhibiting neither a morphological nor a substantial change until they are finally cast out of the sperm. But under abnormal conditions, such as when the animal was underfed or completely starved, it was observed that the granules steadily decreased in number until they almost completely disappeared from the cell. When, after a few days of starvation, food was again restored to the insect the granules were seen to reappear as gradually as they had disappeared, except in those cells where disorganization induced by the general physiological retrogression had advanced too far. The gradual disappearance of the granules suggests that they possibly constitute a stock of reserve material with a nutritive function, and in order to counteract the effects of prolonged starvation the cell is probably making a demand on this reserve.

The characteristic and uniform behaviour of the granules during the maturation stages of the cell in almost every instance where they have been observed—a behaviour which ensures

an almost equal division of the granules between the daughter cells—probably lends support to the view that they constitute a stock of reserve material with a nutritive function, the purpose of the division being to provide the daughter cells with a supply of reserve material. The behaviour of the granules is one of those interesting phenomena the exact mechanism of which is hard to explain. In all probability as suggested by Gatenby (5), such a division of the granules between the daughter cells is brought about by cytoplasmic currents.

Referring to the Y-granules, Parat and Villela have stated in a comparatively recent paper (26) that they consider them to be composed of 'Krinom' material. But 'Krinom' as accepted by cytologists is a new substance formed in the cell by the combination of certain cell substances with neutral red. The Y-granules are not new formations, but pre-existing bodies often visible in fresh preparations without the application of neutral red. Gatenby and Duthie, in discussing the claims of Parat and Villela, have (10) cited all those instances on record where the Y-granules were observed, without the aid of neutral red, in fresh preparations. In view of this fact, there is no justification for identifying the Y-granules with the 'Krinom' of Chlopin.

#### SUMMARY.

1. Recent researches have established that the Y-granules exist as a fundamental part of the constitution of the male germ-cells of many animals, vertebrates as well as invertebrates.

2. Prior to the application of vital staining techniques to cytological problems, *Saccocirrus* was the only animal in which these granules were known to exist.

3. The fixatives generally used by cytologists, especially those containing acetic acid and other fat solvents, are not indicated for studying the Y-granules. Vital staining techniques offer the best method for their study.

4. Underfeeding and starvation of the larvae of *Agrotis segetum* and *Abraxas grossulariata* revealed that these granules are products of normal metabolic activity, and that their appearance and disappearance depend on the general physiological status of the animal.



5. In normally fed larvae the Y-granules were invariably present, and responded to the vital dye within a few minutes of its application. The germ-cells of underfed larvae showed a steady decrease in their Y-granule contents, as did starved larvae up to a certain stage. When starvation was prolonged the granules disappeared from the cell.

6. The changes produced by underfeeding and starvation in the cell, both in the Y-granule content and the colloidal state of the cytoplasm, were decidedly more pronounced in the spermatocytes than in the spermatids. In very advanced stages of the cell scarcely any change was perceptible.

7. The chemical composition of the granules was determined by studying their reactions to certain vital dyes and fixatives. Neutral red, brilliant Cresyl blue, and Nile-blue sulphate were successfully used to stain the granules, Nile-blue sulphate being particularly satisfactory. This stain is specific for fats and substances chemically allied to fats.

8. Y-granules are composed of either fatty acids or a mixture of fatty acids and neutral fat, because they always stain blue with Nile-blue sulphate.

9. Fixatives containing osmic acid failed to stain the granules except in rare cases when they appeared brown in the preparations. As a rule they appeared a shade of pale grey. As this is a reaction for saturated fatty acids it is believed that in the majority of cases where in fixed preparations the granules are seldom visible, they are composed of saturated fatty acids.

10. The fatty nature of the material was confirmed by fixing testis smears in formalin vapour, and staining them with Herxheimer's solution of acetone and scarlet R. If the preparations are counterstained in methylene blue the Y-granules are clearly seen stained pink—a reaction indicating the fatty acid nature of the granules.

11. An examination of the ovaries of *Abraxas grossulariata*, *Gryllus domesticus*, and *Lithobius forficatus*, supravitally stained in neutral red, revealed in every case a juxtannuclear aggregate of neutral red staining granules. With the growth of the oocytes the granules wandered into the cytoplasm and gradually developed into small spheres; their

substance simultaneously underwent a chemical change. By the aid of Nile-blue sulphate it was possible to ascertain that the fatty acid contents of the granules gradually changed into fat, or rather into fatty yolk.

12. The behaviour of the granules in the oocytes suggest that the Y-granules in the spermatocytes are only abortive yolk granules having no function under normal conditions. But a gradual reduction in their number, observed in several spermatocytes when the animals were starved, suggests that under exceptional circumstances there is a possibility of the granules exercising some sort of storage function.

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## DESCRIPTION OF PLATE 27.

## ABBREVIATIONS.

F.E., follicular epithelium.	o., oocyte.
g., Golgi bodies.	v., vacuole.
gm., germarium.	w., wall of ovariole.
m., mitochondria.	y., yolk spheres.
m.N., mitochondrial nebenkern.	y.G., Y-granules.

Fig. 1.—Normal spermatocyte from a regularly fed larva of *Agrotis segetum*.

Fig. 2.—Spermatocyte from a starved larva of *Agrotis*. Note the great reduction in the Y-granule contents and the vacuoles in the cytoplasm.

Fig. 3.—A spermatid of *Abraxas grossulariata*.

Fig. 4.—Young oocyte of *Abraxas* showing two groups of juxtanuclear aggregations of neutral red staining granules.

Fig. 5.—An older oocyte of *Abraxas*.

Fig. 6.—Ovariole of *Gryllus domesticus*.

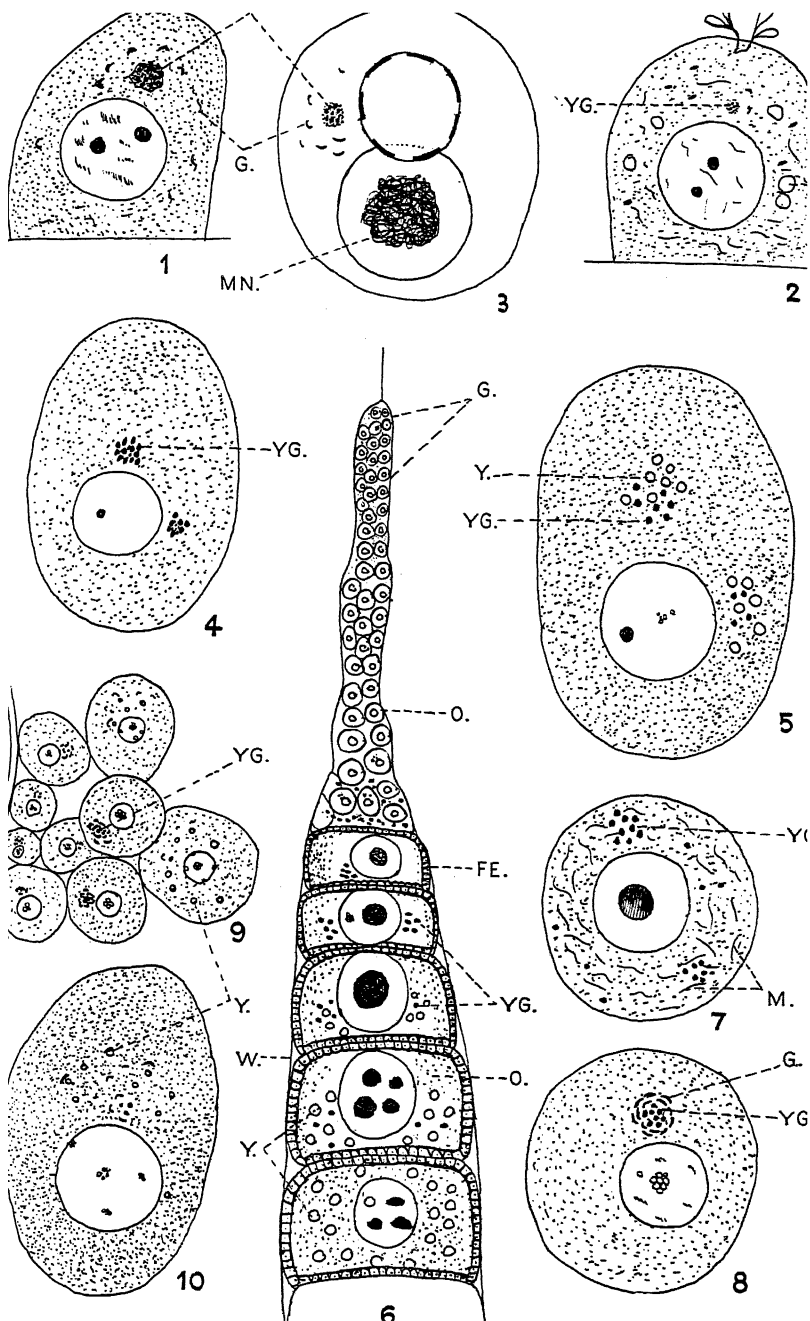
Fig. 7.—Spermatocyte of *Lithobius forficatus*.

Fig. 8.—Young oocyte of *Lithobius forficatus*.

Fig. 9.—A part of the entire ovary of *Lithobius forficatus*.

Fig. 10.—An older oocyte of *Lithobius*.







**The Germinal Layers concerned in the Formation  
of the Alimentary Canal and Malpighian  
Tubules of *Ephestia Kühniella* (Lepidoptera).**

By

**Mabel Drummond,**

Imperial College of Science and Technology.

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With Plates 28 and 29.

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INTRODUCTION.

THE origin and nature of the mid-gut epithelium of insects is of particular interest because of the differences of opinion which have existed with regard to this aspect of insect embryology.

Eastham (1), in his review and in his work on *Pieris*, has shown that many of the conflicting views may be reconciled since they are due to differences of interpretation, and in some cases to faulty technique, rather than to actual variations in development.

Of the recent work mention must be made of Mansour's on *Calandra oryzae*, Johannsen's on *Diacrisia virginica*, Eastham's on *Pieris rapae*, and Henson's on *Pieris Brassicae*.

Mansour (4) describes the mid-gut epithelium as arising from the blind ends of the stomodaeum and proctodaeum and considers it to be ectodermal. This view was held by Heymons (1895) and by other workers since that time.

Johannsen (3) considers the mid-gut epithelium to be endodermal. The endoderm rudiments first appear at the blind end of the stomodaeum and proctodaeum.

Eastham (1) and Henson (2) have found that in the genus *Pieris* the mid-gut epithelium is endodermal, and arises from anterior and posterior rudiments which are proliferated from the germ-band independently of the stomodaeal and proctodaeal invaginations.



Until the work of Henson, the malpighian tubules of insects, when associated with the proctodaeum, have been on account of their position regarded as ectodermal.

Henson suggests that the stomodaeum and proctodaeum are not purely ectodermal. They arise on the site of the anterior and posterior rudiments which have similar positions to those occupied by the embryonic mouth and anus of *Peripatus*. He believes that these regions in insects and in *Peripatus* are homologous. In *Peripatus* these openings into the gut remain after the closure of the middle portion of the blastopore and are carried inwards by the developing stomodaeum and proctodaeum. Accordingly, the blind ends of the stomodaeum and proctodaeum of insects are not ectodermal 'but are composed of tissue homologous with the lips of the embryonic mouth and anus of *Peripatus* (i.e. the blastopore lips)'.

In the larva two rings of cells can be distinguished which separate the ectoderm of the fore- and hind-gut from the endoderm of the mid-gut. These rings, the interstitial rings of the canal, are not visible during embryonic development. A ring similar to the proctodaeal interstitial ring separates the main duct of the malpighian tubules from the tubules proper. Henson expresses the view that these rings are the remains of the blastoporic areas which during development covered the tips of the stomodaeum and proctodaeum. He draws attention to the fact that the interstitial rings are in the same position as the lips of the blastopore in *Peripatus*. The interstitial ring of the tubules may then be due to the carrying away by the tubules of a portion of the blastopore lip during their development. He concludes, therefore, that the tubules proper are endodermal, the ring marking the end of the ectoderm. They would then be homologous with the tubules which in some insects arise from the mid-gut.

#### MATERIAL AND METHODS.

As mentioned later, I procured an abundant supply of material of the moth *Ephestia kühniella* Zeller from Professor Munro.

The females, which prefer to lay their eggs in the dark, were

enclosed in tubes lined with black paper, and upon this the eggs were deposited. The eggs were pricked and preserved in Bouin's fixative. After fixation they were stained in borax carmine, dehydrated and cleared in mixtures of absolute alcohol and benzene. The eggs were then placed on the top of the embedding oven in a mixture of wax and benzene and left overnight, after which they were embedded in pure wax.

Sections were cut  $5\mu$  thick, and stained in Delafield's haematoxylin.

#### DEVELOPMENT.

The early development of *Ephestia* closely resembles that of *Pieris rapae*, and only a brief description will be given.

Repeated divisions of the zygote nucleus give rise to cleavage nuclei which migrate towards the periphery and enter the cortical layer of the cytoplasm to form the blastoderm and serosa. Certain of the nuclei remain in the yolk as yolk nuclei. The blastoderm thickens to form the germ-band which, by decrease in width, comes to occupy the ventral region of the egg. Extension of the serosa takes place as a fold overlapping the blastoderm, the inner side of the fold being formed by the amnion, and this causes the germ-band to sink into the yolk. Growth in length of the germ-band now takes place by flexure of the anterior and posterior extremities of the band towards the dorsal surface of the egg.

Gastrulation.—Along the median line of the germ-band with the exception of the cephalic and caudal extremities, a thickening takes place so that a middle plate becomes differentiated from the two lateral regions. This middle plate is invaginated into the yolk and is enclosed on the ventral surface by the overgrowth of the lateral regions. It now lies against the yolk as the body mesoderm which quickly becomes divided into segmental and inter-segmental groups of cells.

During the invagination of the middle plate the evanescent endoderm cells are given off into the yolk, and there disintegrate.

These cells have been noted in *Pieris* and *Calandra*. In *Diacrisia*, however, there is no proliferation of endoderm at the time of gastrulation, but this occurs later at the time of formation of the neural groove.

In the cephalic and caudal extremities in which mesoderm formation does not take place in the ordinary way, there are two masses of cells which are formed by budding from the germ-band at the time of invagination of the middle plate. These are the cephalic and caudal mesendoderm rudiments (fig. 1, Pl. 28, and fig. 10, Pl. 29).

These rudiments occur in *Pieris*, but are not present in *Calandra*. In *Diacrisia* there are two masses of cells which are the extremities of the band of median endoderm, but which later break down.

Whereas in *Pieris* the cephalic region of the germ-band curves through  $180^\circ$ , that of *Ephestia* curves through at least  $360^\circ$ , so that the extremity is directed anteriorly or even entero-dorsally. Moreover, on account of the great increase in length, the flexure is in the form of a spiral.

*Stomodaeum*.—The stomodaeal invagination begins opposite the cephalic mesendoderm rudiment and, according to the degree of flexure, is at first directed dorsally or, dorso-posteriorly. As the invagination deepens the cells of the rudiment are carried with it. The cells proliferate and those destined to form cephalic mesoderm spread towards the base, while those which will form endoderm spread over the blind end (fig. 2, Pl. 29). Segmentation of the body which was indicated at the time of gastrulation has now become marked. The segments are slightly telescoped, thus allowing the embryo to straighten, so that the stomodaeum is carried through  $90^\circ$  and is directed anteriorly. During this time the invagination is deepening and the endoderm becomes displaced to the ventral border of the blind end (fig. 3, Pl. 28). It is from this endoderm rudiment and the corresponding proctodaeal one that the whole of the mid-gut epithelium is formed.

In *Calandra*, as previously stated, the cells of the mid-gut epithelium are proliferated directly from the blind ends of the stomodaeum and proctodaeum. In *Diacrisia* the endoderm rudiments are differentiated later than those of *Pieris* and *Ephestia*, and first appear at the lateral angles of the stomodaeum and proctodaeum.

*Oesophageal Valve*.—During this time also the develop-

ment of the oesophageal valve begins. The valve of *Pieris* has been described by Henson. The blind end of the stomodaeum swells out and the stalk pushes into it giving it the form of a mushroom. The stalk projects into the lumen of the blind end in the form of three folds, two of which later elongate to form the valve of the larva.

The oesophageal valve of *Ephestia* differs from that of *Pieris* in that it is formed from six tubules. While the stomodaeal invagination is becoming directed anteriorly two groups of three tubules grow out from the blind end. Their lumina are continuous with the lumen of the blind end. The three tubules of a group arise from a common point of origin and grow backwards over the mesoderm surrounding the stomodaeum towards the opening of the latter. The tubules respectively bear a ventral, right and left ventro-lateral, dorsal and right and left dorso-lateral relationship to the stomodaeum. The centre tubules of the groups are ventral and dorsal respectively (fig. 6, Pl. 28).

The endoderm rudiment which was on the ventral border of the blind end of the stomodaeum becomes divided into two groups of cells placed ventro-laterally and connected together by a fine strand of cells. The growth of the valve tubules pushes the endoderm rudiment backwards from the blind end of the stomodaeum. The two groups of cells remain connected together and to the tip of the stomodaeum (fig. 4, Pl. 28). From each group also a fine strand of cells is being proliferated, and is extending anteriorly under cover of the mesoderm of the anterior body segments (fig. 5, Pl. 28). These strands then extend ventrally and finally posteriorly to meet similar strands proceeding from the proctodaeal rudiment. From these cells the mid-gut epithelium is formed.

Further straightening of the embryo now takes place, and the opening of the stomodaeum is carried through a dorsal to an antero-dorsal position. The blind end is directed ventro-posteriorly and becomes very thin. The lumen is evaginated between the valve tubules. During formation of the mid-gut epithelium the blind end of the stomodaeum is overgrown by endoderm and, as will be mentioned later, the fine ectodermal

lamina of the blind end breaks down. This lamina formed the outer parts of the walls of the valve tubules and, upon its breaking down, the inner parts of the walls remain lying in the recesses between the evaginations of the stomodaeal wall (fig. 7, Pl. 29).

After closure of the dorsal wall of the embryo the oesophageal valve begins to elongate posteriorly by a growth of the tubule walls together with the stomodaeal wall. The valve presses against the endodermal lamina which covered the blind end of the stomodaeum, and this ultimately breaks down. The valve now extends for some distance into the cavity of the mid-gut (fig. 8 and fig. 9, Pl. 29). The areas of junction between the endoderm and the valve become closely applied to the wall of the fore-gut, and the valve is enclosed and is not visible externally on the gut of the larva.

Proctodaeum.—The posterior region of the germ-band curves through  $270^\circ$  and is in the shape of a spiral. The caudal mesendoderm rudiment at first lies in the arch formed by the germ-band in curving from  $180^\circ$  to  $270^\circ$  (fig. 10, Pl. 29). The proctodaeal invagination begins opposite the rudiment, and continues in a ventral or ventro-posterior direction (fig. 11, Pl. 29). As the embryo straightens the proctodaeum becomes directed ventro-anteriorly. It deepens rapidly, and the blind end becomes very thin. The endoderm rudiment is small and, like the anterior mesendoderm rudiment, lies on the ventral border of the blind end (fig. 12, Pl. 29). From the rudiment two strands of cells pass anteriorly as already mentioned.

The first rudiments of the malpighian tubules are now given off ventro-laterally as lobes from the blind end of the proctodaeum, and the endoderm rudiment overlies these (fig. 13, Pl. 29). Two main ducts from the proctodaeum each quickly divide into three tubules.

The embryo straightens further. The proctodaeal invagination deepens and for a time the blind end remains thin. The backward growth of the malpighian tubules carries the endoderm backwards from the tip of the proctodaeum (fig. 14, Pl. 29). The proctodaeum continues to deepen and the blind end begins to thicken. Forward growth in this region now leaves the opening of the malpighian duct slightly behind the tip.

During this time the endoderm has enclosed the yolk on the ventral surface, and only the dorsal surface of the embryo remains to be completed. The abdominal segments, which are the first to be completed dorsally, sink downwards and backwards so that the opening of the proctodaeum is carried through a posterior to a ventral position, and the flexure of the embryo changes from a dorsal to a ventral one. The opening of the stomodaeum becomes ventral and its blind end lies opposite the yolk passage, and for a time is in contact with the yolk present between the amnion and serosa. The endoderm grows dorsally to complete the mid-gut, and grows over the blind ends of the stomodaeum and proctodaeum. The ectoderm of the stomodaeum then becomes continuous with the endoderm of the mid-gut.

In *Pieris* the blind ends of the stomodaeum and proctodaeum are said to assume an endodermal character.

In *Ephestia* the very fine ectodermal lamina of the stomodaeum breaks down after the endoderm has overgrown it (fig. 15, Pl. 29). An endodermal lamina is thus left separating the lumina of the fore- and mid-gut.

The blind end of the proctodaeum and the overlying lamina of endoderm separate the mid-gut lumen from that of the hind-gut (fig. 16, Pl. 29). These two laminae break down just before hatching.

#### CONCLUSIONS.

The development of the mid-gut epithelium of *Diacrisia* differs from that of *Ephestia* merely in the time of appearance of the endoderm rudiments relative to the invaginations of the stomodaeum and proctodaeum. Eastham and Johannsen have both stated that the variations observed in different embryos may be explained in this way. It may be that the endoderm rudiment of *Diacrisia* is derived from the anterior endoderm mass which Johannsen observed breaking down, since, both in *Ephestia* and *Pieris*, cells are given off into the yolk from the apex of the stomodaeum.

With regard to the alleged ectodermal nature of the mid-gut epithelium of *Calandra*, it should be noted that the endodermal rudiment may be so small as to be readily mistaken for a proliferation from the ectoderm as, for example, the proctodaeal

rudiment of *Ephestia*. The endoderm cells are distinguished from those of the ectoderm by differences of appearance which are largely dependent upon the fixative used.

In view of the fact that the blind ends of both stomodaeum and proctodaeum are ectodermal, the conclusion that in *Ephestia* these tissues are homologous with the blastopore lips of *Peripatus* would be erroneous.

The malpighian tubules, since they arise from a purely ectodermal structure, namely the proctodaeum, are therefore essentially ectodermal.

#### ACKNOWLEDGEMENTS.

I wish to thank Professor E. W. MacBride, F.R.S., for suggesting the problem and for his help and encouragement. I would also thank Professor J. W. Munro, D.Sc., for supplying me with material.

#### SUMMARY.

1. The mid-gut epithelium of *Ephestia* is derived from cephalic and caudal mesendoderm rudiments which are proliferated from the germ-band independently of the stomodaeal and proctodaeal invaginations.

2. The oesophageal valve is formed by the backward growth from the blind end of the stomodaeum of six tubules, which later in a slightly altered form extend into the cavity of the mid-gut to form the valve of the larva.

3. The stomodaeum and proctodaeum are purely ectodermal.

4. The malpighian tubules are ectodermal.

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## EXPLANATION OF PLATES 28 AND 29.

## ABBREVIATIONS.

*Amn.*, amnion; *B.C.*, blood-cell; *B.End.*, endoderm covering blind end of stomodaeum; *B.Stom.*, blind end of stomodaeum; *C.Mes.Rud.*, cephalic mesendoderm rudiment; *Cau.Mes.Rud.*, caudal mesendoderm rudiment; *Ect.*, ectoderm; *End.*, endoderm; *Gen.Rud.*, genital rudiment; *H.G.*, hind-gut; *Haem.*, haemocoel; *Malp.*, malpighian duct; *Mes.*, mesoderm; *M.G.*, mid-gut; *M.G.End.*, mid-gut endoderm; *Proc.*, proctodaeum; *R.V.T.*, remnant of valve tubule; *S.Mes.*, somatic mesoderm; *Ser.*, serosa; *Stom.*, stomodaeum; *Sub.oes.b.*, sub-oesophageal body; *V.T.*, valve tubule; *W.Stom.*, wall of stomodaeum; *Y.C.*, yolk-cell.

## PLATE 28.

Fig. 1.—Cephalic region of germ-band with cephalic mesendoderm rudiment. Sagittal section.  $\times 600$ .

Fig. 2.—Stomodaeal invagination with mesoderm on the sides and endoderm covering the blind end. Sagittal section.  $\times 400$ .

Fig. 3.—Stomodaeal invagination with endoderm on the ventral border of the blind end. Sagittal section.  $\times 400$ .

Fig. 4.—Stomodaeal invagination after the outgrowth of the valve tubules, two of which are shown. The endoderm is carried backwards by the growth of the tubules. Frontal section.  $\times 400$ .

Fig. 5.—Stomodaeal invagination and one valve tubule. A fine strand of endoderm is growing anteriorly to meet a similar strand from the proctodaeum. Sagittal section.  $\times 400$ .

Fig. 6.—Stomodaeum and six valve tubules. Transverse section.  $\times 400$ .

Fig. 7.—The oesophageal valve after endoderm has covered the blind end of the stomodaeum. The ectodermal covering of the blind end has broken down and the remnants of the tubule walls are lying in recesses of the stomodaeal wall which has become evaginated between the tubules.  $\times 400$ . Transverse section.

Fig. 8.—The oesophageal valve just before hatching of the embryo. Transverse section.  $\times 800$ .

Fig. 9.—Oesophageal valve after elongation into the cavity of the mid-gut. Four parts of the valve are shown. Sagittal section.  $\times 400$ .

## PLATE 29.

Fig. 10.—Caudal region of the germ-band with caudal mesendoderm rudiment. Sagittal section.  $\times 600$ .



Fig. 11.—Proctodaeal invagination with mesoderm on the sides and endoderm covering the blind end. Sagittal section.  $\times 400$ .

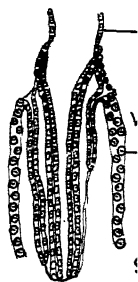
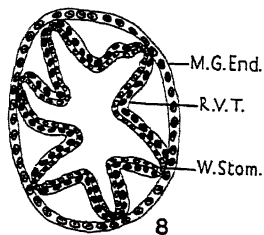
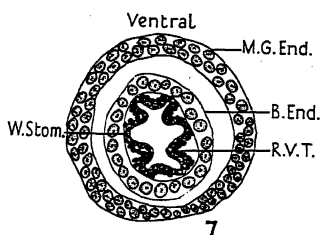
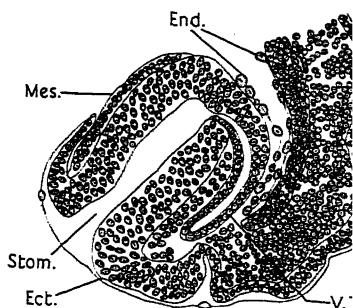
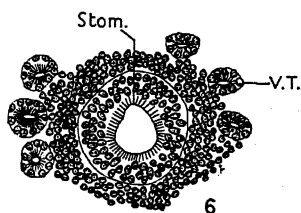
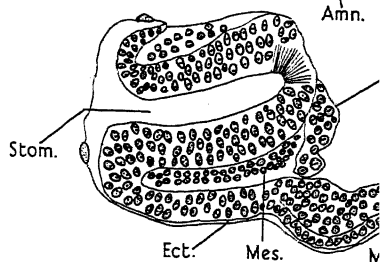
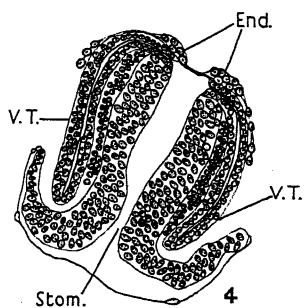
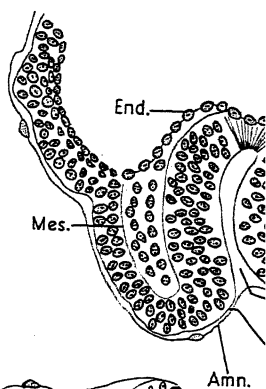
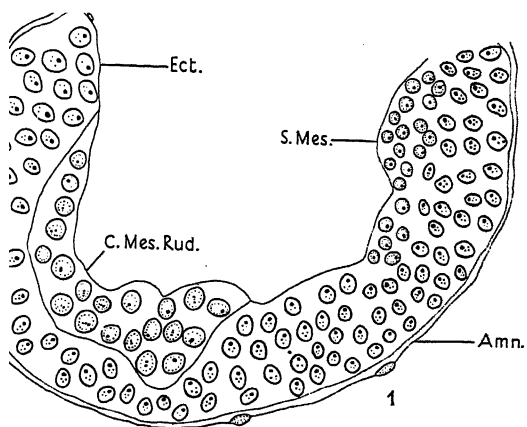
Fig. 12.—Proctodaeal invagination with endoderm on the ventral border of the blind end. A strand of endoderm is growing forwards. Sagittal section.  $\times 400$ .

Fig. 13.—First rudiment of the malpighian tubules. Sagittal section.  $\times 400$ .

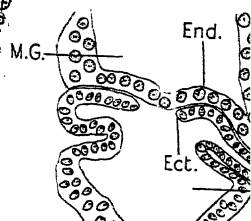
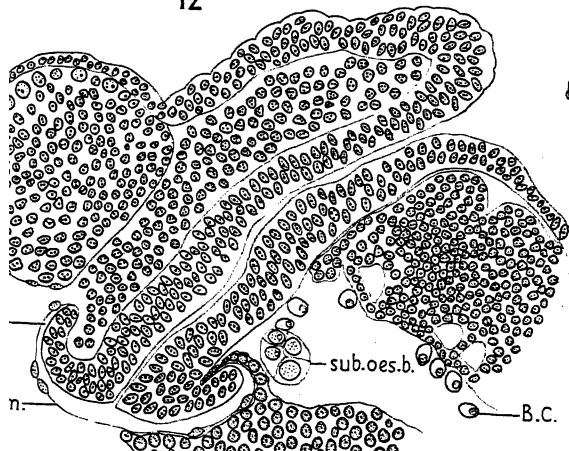
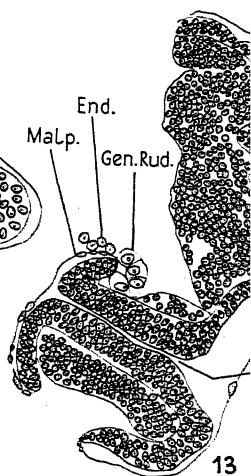
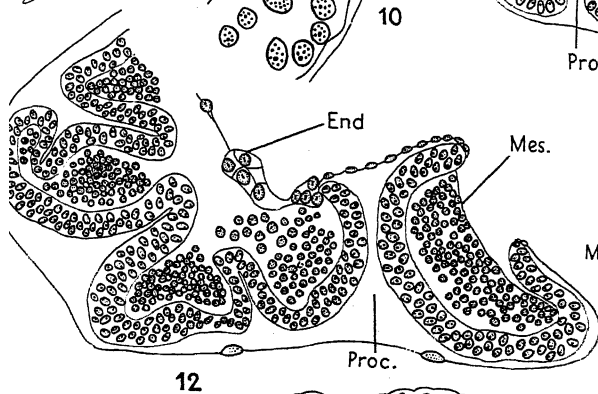
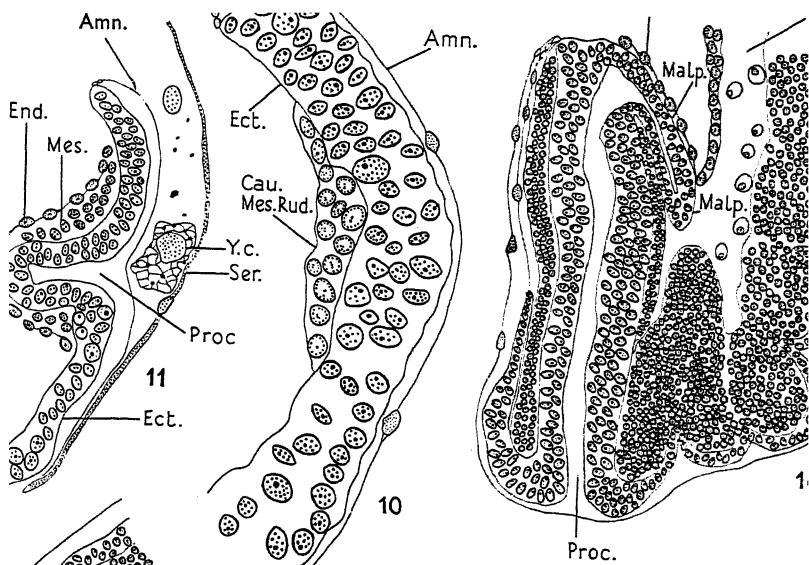
Fig. 14.—Later stage of the proctodaeal invagination. The growth of the malpighian duct carries the endoderm which overlies it backwards. Sagittal section.  $\times 600$ .

Fig. 15.—Stomodaeal invagination with ectodermal lamina covering the blind end. Endoderm is growing over this lamina, which later breaks down, so leaving an endodermal lamina separating the cavities of the fore- and mid-gut. Sagittal section.  $\times 600$ .

Fig. 16.—Mid- and hind-gut just before hatching of the embryo. An ectodermal and an endodermal lamina separate these cavities, and both break down prior to hatching. Sagittal section.  $\times 400$ .









# The Development of the Sabellid *Branchiomma vesiculosum*.

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With 30 Text-figures.

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## HISTORICAL INTRODUCTION.

IN spite of the many species included in the Polychaete family Sabellidae, their world-wide distribution and their frequent local abundance, only two short papers describing their larval forms have so far been published. The more recent of these is fifty years old (Roule, 1885). In both the larvae described were those of *Dasychone lucullana* Delle Chiaje. Larvae of this same species were later used by Calgreen (1900) in a study of the influence of constant galvanic currents

on various invertebrate organisms; he found that they were kathodically galvanotactic. He obtained the larvae at Naples from egg-capsules laid by the adults, but only gives a very brief description of them. The earliest account of *Dasychone* larvae was by Claparède and Mecznirow (1868). It was short but was illustrated by a number of coloured figures. They found the eggs enclosed in a mucus sac shaped like a thick ring around the tube of the parent. The yolky larvae, with two eye-spots and a strong prototroch, were freed from the mucus sac. The body elongated, bristles appeared, and the branchial lobes developed anteriorly to the prototroch. The larvae metamorphosed and the young worms grew for some time afterwards. On the whole the development was evidently very similar to that of *Branchiomma vesiculosum* Montagu here described.

Roule's paper is without figures and is merely an outline sketch of the external features of the development observed at Marseilles. Like his predecessors he obtained his larvae from the hatching out of mucus-embedded eggs spread round the opening of the adult tube. The branchial rudiments were again seen to develop in front of the prototroch. On metamorphosis the larvae attached themselves by their posterior extremities to the sides of the vessel in which they were reared. These extremities were well provided with mucus cells and this mucus was used for fixation. Subsequently a transparent tube was secreted. At the time of settling the larvae were stated to be one to two millimetres long, which makes them several times the length of *Branchiomma* larvae at the corresponding stage. From the few details given of the metamorphosis it is not possible to say whether the larval head tissue is cast off, as in *Branchiomma*, or absorbed internally. Bristles and uncini appear, and one gathers that, as in *Branchiomma*, there is no change over of anterior segments from the abdominal to the thoracic type during normal development. Six weeks after hatching the young worms had about twelve segments and apart from their size possessed all the main adult features. They were then five to six millimetres long, which again makes them much bigger than corresponding juveniles of *Branchiomma*.

Although *Branchiomma* is kept alive in captivity in the

Plymouth Aquarium all the year round, and lives for many months without showing signs of ill health, I have never known them to spawn there. Hornell (1893), however, observed this activity in the aquarium of the Jersey Station, and he describes how the ova were discharged freely into the water. It seems that they were passed up from below into the funnel formed by the filaments until a quantity had there accumulated, a sudden retraction of the animal then shooting them upwards and outwards. The process was repeated more or less regularly for nearly three-quarters of an hour. Almost simultaneously with this a male emitted a cloud of sperm that rose slowly upwards amidst its filaments, but in this sex there was no ejection by retraction. Other individuals in the same aquarium followed suit, and all had finished spawning by the end of the day. This interesting observation proves that *Branchiomma* does not lay its eggs enclosed in mucus as does *Dasychone*. I have removed many hundreds of *Branchiomma* from their tubes, and never observed anything resembling a cluster of eggs enclosed in mucus. Hornell has nothing to say about the development of the eggs.

Brunotte (1888) describes how he discharged the eggs and sperm of *Branchiomma* through the pores of the segmental organs by pressing lightly on the body-wall. He did not observe the natural spawning nor, apparently, the development of the eggs.

#### METHODS.

The adult worms used in making the artificial fertilizations were dug out of muddy gravel near low-water mark of spring tides on the south-eastern slopes of the Salstone, situated up the Kingsbridge estuary, distant more than two miles from the open sea. A description of this ground can be found in Allen and Todd (1900). For assistance on many occasions in the heavy work of digging up the worms the writer wishes to record his grateful thanks to Mr. William Searle. The worms lived well under circulation in the laboratory and could be used a considerable time after collecting. Many early attempts to fertilize the eggs were unsuccessful, but it was eventually discovered that if,



instead of employing clean methods with relatively few eggs and little sperm—methods that give excellent results with many other species—thirty or forty worms, male and female, were slit open and the contents of their body cavities washed out into a bowl containing relatively little sea-water, larvae would be obtained during the summer months. The thick suspension of eggs and sperms was thoroughly mixed by stirring and, after ten minutes to half an hour, the excess sperm was washed away from the eggs (which sank to the bottom of the dish) by frequently decanting with fresh sea-water. The eggs were then distributed between a number of flat-bottomed glass finger-bowls so that they lay on the bottom not more than one layer thick. By the next morning a few larvae would be found swimming near the surface, especially round the edge of the meniscus. The majority of the eggs were always unsegmented, and in the last fertilizations the larvae represented only a fraction of one per cent. of the total number of eggs used. Swimming larvae were pipetted off to clean filtered sea-water in other finger-bowls, where they developed and metamorphosed without further trouble. A few drops of a culture of the diatom *Nitzschia* were added to each bowl, but food was not taken while the larvae were free-swimming. At metamorphosis they settled on the sides and bottom of the bowls, where they usually grew for several weeks.

The first successful fertilizations were made in June and July 1929. Further fertilizations were made in May, June, and July 1932, May and June 1933, and in July 1934. Altogether larvae were reared many times.

For histological work larvae were fixed in Bouin's fluid at 60° C., which was found to give very satisfactory results. Larvae contracted slightly on fixing, but the rapidity with which the hot fixative killed stopped undue contraction. Over two hundred and thirty larvae and young worms were sectioned in three planes. Relatively exact orientation was obtained by my method described elsewhere (Wilson, 1933). The process, although rather long and tedious, gave results that justified its use. Sections were cut at 4 $\mu$ , 6 $\mu$ , and 8 $\mu$ , the most generally useful thickness being 6 $\mu$ . The stains mainly employed were

Heidenhain's iron-alum haematoxylin and Delafield's haematoxylin.

Whole mounts were made in various ways. Specimens stained with alum carmine and mounted in Canada balsam and unstained specimens mounted in Euparal were most useful.

With the exception of the chaetae all the drawings were made using a squared net micrometer in the eyepiece, drawing first on to squared paper. The chaetae were drawn with an Abbé camera lucida. The drawings of whole larvae and young worms were made from life while they were confined, but not compressed, in cavity slides.

Interpretation of the sections has often been very difficult on account not only of the minute size but also because of the yolk and compact nature of the tissues, the narrowness of the cavities, and the general absence or indefiniteness of cell limits. For these reasons some details have not been made out and some interpretations are still doubtful, as will be indicated in the appropriate places. The drawings of sections are all careful copies of original sections, checked on several similar sections of other individuals. They are necessarily somewhat diagrammatic, for it is not possible to convey in black lines and dots the delicate tones of the actual tissues. In particular, little attempt has been made to illustrate fine cytoplasmic details, and the cytoplasm is for the most part conventionally dotted.

#### GENERAL ACCOUNT OF EARLY DEVELOPMENT.

The newly shed eggs are brown by reflected light. They are very opaque and granular with large germinal vesicles. They have a diameter of approximately  $150\mu$  and are enclosed in a membrane of about  $3\mu$  thick. This membrane consists of at least two layers, and during early cleavage stages it can be seen that while the outer layer is smooth the inner is slightly wrinkled, so that minute cavities are produced between the two, giving a slightly 'vesicular' appearance to the membrane as a whole. An attempt to indicate this appearance has been made in Text-fig. 1 A and B. Polar bodies are produced after fertilization, and in early cleavage stages can be seen attached to one of the blastomeres inside the egg membrane.

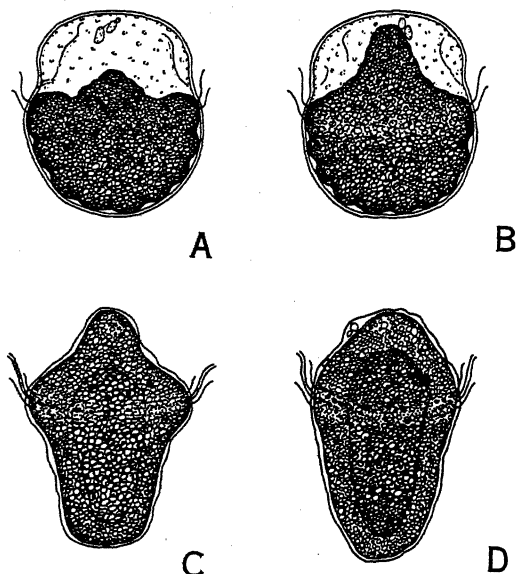
When the larvae first begin feebly to swim they are of the curious shape shown in Text-fig. 1 A. They are almost hemispherical, filling one-half of the cavity inside the egg membrane. The cells round the edge of the hemisphere form the prototroch, which at this stage possesses two narrow bands of fine cilia that pass through the egg membrane. The cilia of the anterior band are longer than those of the posterior. These bands are not continuous all round, there being a slight gap in one place, which subsequent development shows to be dorsal. On the flat surface of the hemisphere a slight pyramid of cells is the rudiment of the head. In the large anterior space between this and the egg membrane two or three clear and irregular fragments are presumably the polar bodies. When larvae at this curious stage were first observed they were mistakenly believed to be abnormal. The rudimentary head in a few hours rapidly enlarges, until it fills up the previously empty space within the egg membrane (Text-fig. 1 B and C). The latter is for a time crumpled, but it is later filled out completely by the growing larva. It is shown by a double line in Text-fig. 1 A and B, a single line in Text-fig. 1 C and D, and thereafter is not specially indicated in the drawings of whole larvae, although it is present as the external cuticle throughout development. It is always indicated in the drawings of sections.

An early stage is shown in dorsal view in Text-fig. 1 C. The head is still growing, the egg membrane over it being very much crumpled. An eye-spot has appeared in the right side. The trunk is elongating and the central endodermal mass is conspicuous. The prototroch, in which there is still a dorsal gap, is definitely the widest region of the body. It has three bands of cilia, an anterior one of short cilia having appeared immediately in front of the two first-formed bands. Cells of the prototrochal region are becoming vacuolated and so is the head vesicle (see below). As a whole the larva is very granular and opaque, especially the endodermal mass.

A twenty-four-hours-old larva is shown in Text-fig. 1 D; it is approximately  $200\mu$  long. The body and head have filled out more than in the previous stage, otherwise it is very much the same. There is still a slight dorsal gap in the prototroch. Two

eye-spots of reddish brown oily looking globules are present, the globules being sometimes grouped irregularly, giving the appearance of two eyes on one side. The cells presumed to be polar bodies are occasionally still seen under the cuticle. The larva swims much more actively than previously.

The larva now elongates and becomes segmented. When three days old (Text-fig. 2) it is about  $330\mu$  long and  $100\mu$  broad at the prototroch. Two of the segments carry notopodial bristles, a pair on each side. The third setiger (chaetigerous segment) is marked out but the bristles do not protrude. There are no uncini. The head is widest in the eye region. Both eyes are deep cups of reddish brown oil globules, filled with a clear, apparently structureless, substance. Sometimes irregular clusters of globules similar to those forming the eyes are present alongside the true eyecups to form supernumerary eyes. At the anterior extremity a clear vesicle, filled apparently with a fluid, is a conspicuous feature. At the extreme tip of the snout there is a small pore in the cuticle, so that at this point only an excessively fine membrane separates the contents of the vesicle from the exterior. A pair of similar, but small, vesicles is present at the extreme posterior end of the body; they are likewise provided with a cuticular pore. In the absence of definite information the function of these vesicles is open to speculation. In abnormal specimens the head vesicle is sometimes absent without any apparent influence on the larva or its development. There are sensory cilia at both ends of the body and a long excessively fine one can occasionally be glimpsed on the back immediately behind the prototroch, in addition also to one on each side posterior to it. The prototroch consists ventrally of five bands of cilia, the second being the broadest and having the longest cilia. In the figures of whole larvae the breadth of this band is indicated at each side by the distance between the two longest lines representing the cilia belonging to it. Other bands are each indicated by a single cilium. The cilia of the fifth band are short and generally project backwards. The fourth and fifth bands are sometimes not complete dorsally. Just posterior to the prototroch ventrally is the minute mouth opening, and leading back from it to the posterior end runs the



TEXT-FIG. 1.

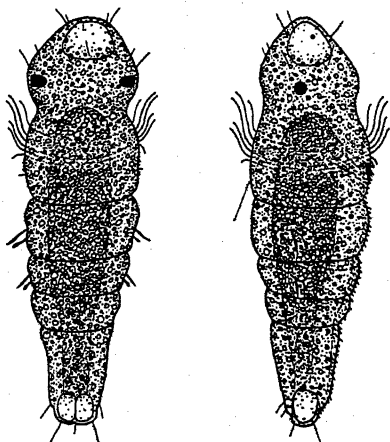
Larvae of *Branchiomma vesiculosum* from life.  $\times 156$ .

A. Embryo when it first swims, about eleven hours after fertilization. B. Embryo an hour or two later. C. Dorsal view of free-swimming embryo about sixteen hours old. D. View of left side of trochosphere about twenty-four hours old. Actual length approx.  $200\mu$ .

#### LIST OF ABBREVIATIONS IN TEXT-FIGURES.

*a.*, anus; *ab.c.*, abfrontal cilia; *a.v.*, anal vesicles; *b.4a.*, swollen base of pinnule 4a.; *b.b.1*, first, &c., bristle bundle; *b.r.*, branchial rudiment; *b.v.*, blood-vessel; *c.*, coelom; *c.g.*, cerebral ganglion; *cir.n.*, circumoesophageal nerve commissure; *c.l.*, collar lobe; *c.m.*, circular muscles; *c.me.b.*, cephalic mesoderm block; *c.r.*, ciliary rootlets; *cu.*, cuticle; *d.c.*, dorsal cilia; *d.f.*, diatom and other food in the gut; *d.l.m.*, dorsal longitudinal muscles; *d.m.*, dorsal mesentery; *d.m.g.*, dorsal mucus gland; *d.pt.*, dorsal gap in prototroch; *e.*, ectoderm; *e.b.*, ectoderm of branchial rudiment; *e.c.*, ectodermal connexion; *e.h.*, ectoderm of adult head; *end.*, endoderm; *ep.*, ectoderm of episphere; *et.*, ectoderm of trunk; *ey.*, eye; *ey.p.*, eye pigment globules; *f.c.*, frontal cell; *h.s.*, head split; *h.v.*, head vesicle; *h.v.c.*, head vesicle cell; *i.*, intestine; *j.1a.*, junction of skeleton of pinnule 1a with pinnule 1; *l.*, ventro-lateral lip of mouth; *l.f.c.*, latero-frontal cilia; *l.h.e.*, larval head ectoderm breaking up; *l.i.*, lumen of intestine; *m.*, mouth; *m.b.b.*, parapodial muscle of bristle bundle; *me.*, mesoderm; *me.b.*,

neurotroch, formed of short cilia apparently contained during life in a very shallow trough. In the mouth region the neurotroch is almost as wide as the body. The tissues of the larva are granular with contained yolk-globules, particularly the walls



TEXT-FIG. 2.

Larvae of *Branchiomma vesiculosum* about three days old, from life.  $\times 156$ . Dorsal view and view of right side. Actual length approx.  $330\mu$ .

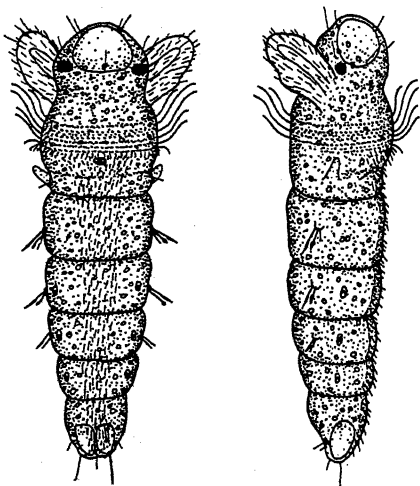
of the gut, which are very opaque. The colour by transmitted light is greenish brown. The larva is contractile and swims actively.

During further growth the body elongates a little and the tissues become less granular and more transparent as the yolk in them is absorbed. Slight swellings appear on each side dorsal to the eye and immediately anterior to the prototroch. They

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mesoderm of branchial rudiment; *m.g.*, mucus gland; *n.*, neurotroch; *n.a.v.*, nucleus of anal vesicle cell; *n.c.*, ventral nerve-cord; *o.*, oesophagus; *p.*, proctodaeum; *p.b.*, portion of a bristle; *p.c.*, problematic cell; *p.c.g.*, central perforation of cerebral ganglion; *pin.*, pinnule; *pt.*, prototroch; *s.1. &c.*, first, &c., septum; *s.c.*, skeletal cell; *sp.*, splanchnopleure; *st.*, stomach; *t.b.*, transverse bar; *v.l.m.*, ventral longitudinal muscles; *v.m.*, ventral mesentery or portion of it; *v.m.g.*, ventral shield gland; *v.v.*, ventral vessel; *y.g.*, yolk-globule.

rapidly enlarge to conspicuous lobes that are destined to branch and give rise to the branchial crown. They early acquire cilia on their ventral surfaces (Text-fig. 3). A small lobe appears on each side a short distance behind the prototroch and above the lateral sensory cilium. They are the rudiments of the future collar. One more setiger has protruded bristles, and uncini have appeared ventral to the bristles in all but the first setiger. In



TEXT-FIG. 3.

Larvae of *Branchiomma vesiculosum* about nine days old and ready to metamorphose. Ventral view and view of right side, from life.  $\times 156$ . Actual length approx.  $355\mu$ .

the stage figured in Text-fig. 3 an uncinus has formed on each side of the fourth setiger, although the bristles do not yet protrude. A certain amount of granular brown pigment has developed in the surface tissues, particularly in the neighbourhood of the prototroch and pygidium.

#### HISTOLOGICAL STRUCTURE OF THE LARVA.

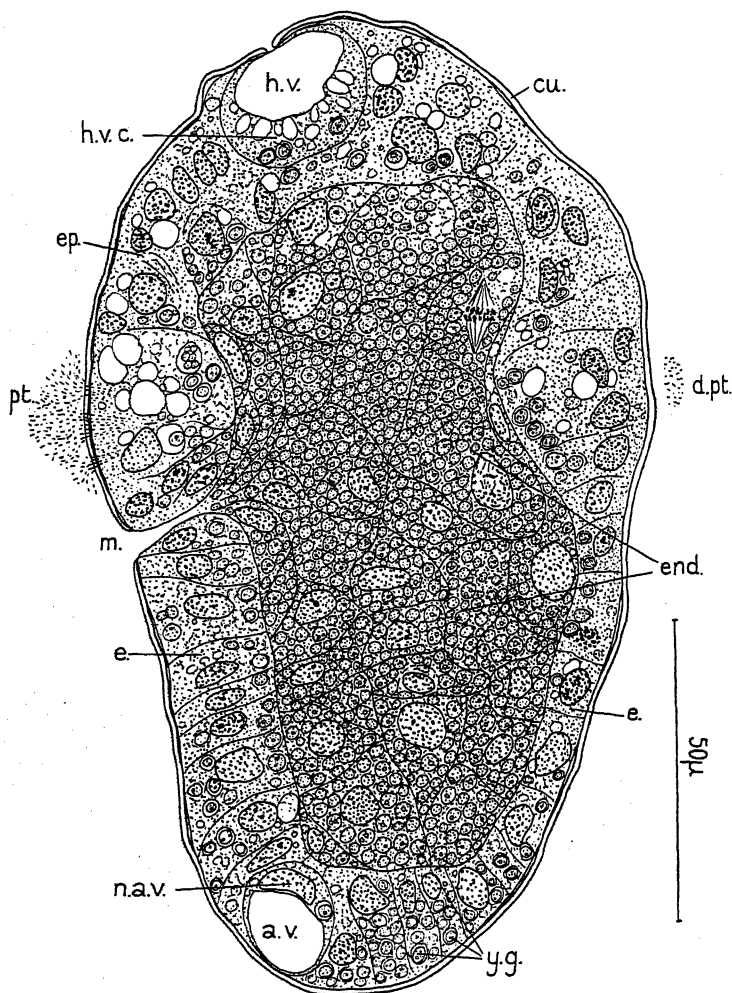
A median sagittal section of an early larva is shown in Text-fig. 4. At this stage few histological details can be distinguished. The outer coat of ectoderm (*e.* and *ep.*) closely surrounds the

central mass of endoderm (*end.*) densely crowded with yolk-globules. The ectoderm also contains yolk-globules but relatively they are not as numerous as those in the gut. With Delafield's haematoxylin some cell outlines can be traced. The nuclei are relatively large and active division stages are taking place in all parts. At the anterior extremity a large cell (*h.v.c.*) is forming in its cytoplasm the very large vacuole or vesicle (*h.v.*) that is such a conspicuous feature of this species. The boundaries of this vesicle cell are relatively clear; it always contains a single large nucleus in the thick layer of cytoplasm behind the vacuole. This nucleus was present in the section adjacent to that from which Text-fig. 4 was drawn. Two cells at the posterior end are likewise becoming vacuolated to form the anal vesicles (*a.v.*). The prototroch cells are large with many fair-sized vacuoles. The cilia can be observed passing through the cuticle, while ciliary rootlets run inwards from their bases. The dorsal gap is, of course, present at this stage. The cuticle (*cu.*) is not too closely adherent to the ectoderm.

The question as to whether or not mesoderm cells are present at this stage has not been decided. The obliteration of the blastocoel and the absence of satisfactory distinguishing marks between the internal cells fails to demarcate them if present. It is, however, quite likely that some of the cells just above the ventral ectoderm are mesodermal.

We have seen that by the third day the definitive form of the larva is marked out. Likewise the various larval organs and tissues become mapped out. It has not been possible to follow this process in detail owing to the general absence of cavities and the minute and yolky nature of the cytoplasm generally, while the cell-walls that were fairly distinct in the earliest stages become less perceptible with increasing age. By the third day, however, it is possible to make out the disposition of the septa, parapodia, longitudinal muscle-fibres, &c., the brain is conspicuous in the ectoderm behind the head vesicle and the longitudinal nerve-cords can be traced. No figures, however, are given of these stages because larvae a few days old show the same structures in a clearer manner, there being no change in essential details between three- and eight-day-old larvae, except for the





TEXT-FIG. 4.

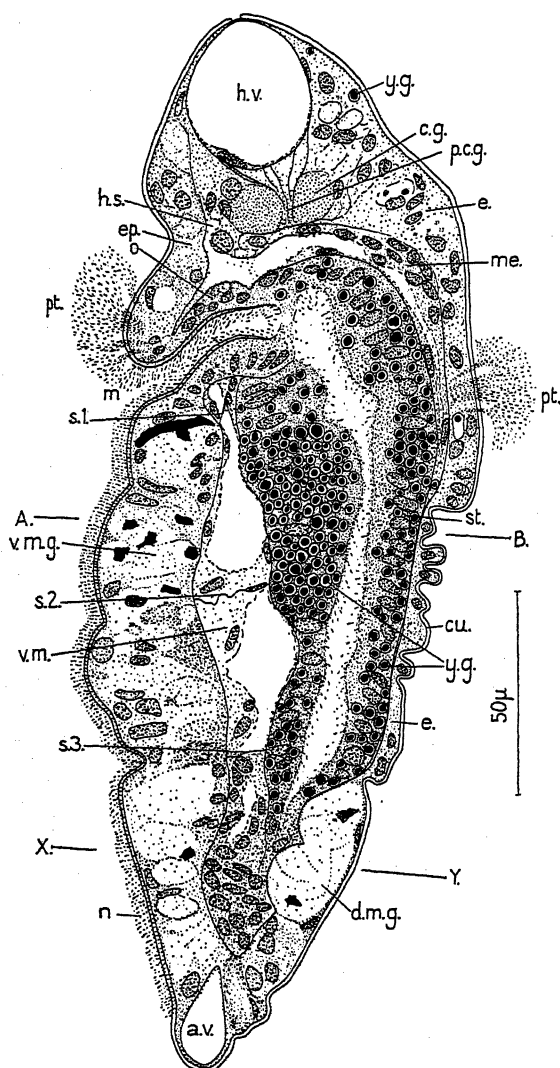
Median sagittal section of a trochosphere about twenty-four hours old. Section stained with Delafield's haematoxylin. For key to lettering see p. 550.

growth of the branchial lobes and the acquisition of one or two more segments. The early larvae have more yolk in all their

tissues and the coelom is much less spacious. Therefore in considering the detailed structure of the last pelagic stages only there will not be missed, so far as is known, any point of importance.

### Ectodermal Structures.

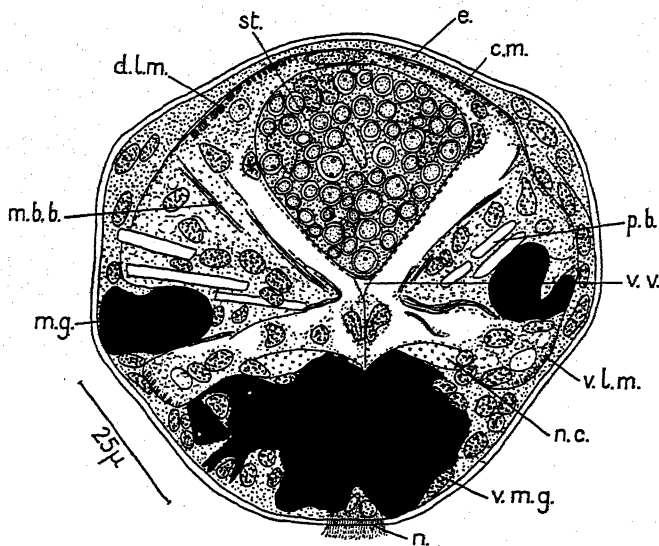
In the epidermis limits between cells cannot as a rule be made out. There is no definite basement membrane, and it is not always easy to trace the boundary between the ectoderm and underlying mesoderm. The epidermis of the trunk is relatively thin with deep transverse furrows dorsally (at least in fixed and presumably slightly contracted specimens), except at the posterior end (Text-figs. 5 and 6, *e.*), but ventrally thickens enormously and has a smoother exterior surface owing to the presence of numerous large mucus secreting cells, which form the ventral shield glands (*v.m.g.*). These cells generally remain unstained with Heidenhain's haematoxylin, except for a few irregular patches that stain an opaque black (Text-fig. 5). With Delafield's haematoxylin they become very dark (Text-fig. 6), and they stain red with Mayer's mucicarmine. In transverse sections one can generally distinguish in the mid-ventral line a plane of demarcation between the glandular cells on either side (Text-figs. 6 and 7). The ciliated band of the neurotroch (*n*) and its ciliary rootlets runs along the mid-ventral line from the mouth almost to the posterior extremity of the body. In the posterior dorsal part of the epidermis, just in front of the position where the anus will open, there is a patch of mucus cells (Text-figs. 5 and 7, *d.m.g.*) similar in size and appearance to those of the ventral shield glands. A large mucus cell is present close by the antero-ventral part of the first parapodium (Text-figs. 6 and 8, *m.g.*), but none so large is associated with the second. Large mucus cells are present ventro-laterally immediately behind the prototroch and there are others present in the epispheral ectoderm (see below) in the ventral half of the head anterior to the prototroch. Just posteriorly to the first parapodium and in front of the second septum a curious cell (possibly two associate cells) is seen (Text-fig. 8, *p.c.*). The cytoplasm with favourable staining by Heidenhain's haematoxylin



TEXT-FIG. 5.

Median sagittal section of an eight-day-old larva ready, or nearly ready, to metamorphose. Heidenhain's haematoxylin.  $\Delta B$ . Approximate plane of section shown in Text-fig. 6.  $\chi Y$ . Approximate plane of section shown in Text-fig. 7.

appears filled with numerous short, dark, rod-like bodies, quite different from the contents of any other cell. The cell does not stain with Delafield's haematoxylin or Mayer's muci-carmin and therefore does not contain mucin. The significance of this paired cell is difficult to elucidate. There is a resemblance in position, size, shape, and possibly also in appearance, to a pair of cells which, following Meyer's description (1888), are present



TEXT-FIG. 6.

Transverse section of first setiger of an eight-day-old larva. Delafield's haematoxylin. Approximate plane of this section shown at AB. in Text-figs. 5 and 8.

in the larva of *Psygmorebranchus* and are the 'Nephridial-schlauchanlagen der bleibenden Thoracalnieren'. In *Branchiomma* I have in vain endeavoured to trace their further history. I have seen them apparently unchanged in a metamorphosed specimen with five to six setigers, but as a rule a short while after metamorphosis they cannot be distinguished. They have not been seen to divide or to give rise to any structure. It may be that, like gland-cells, they discharge their contents and disappear.

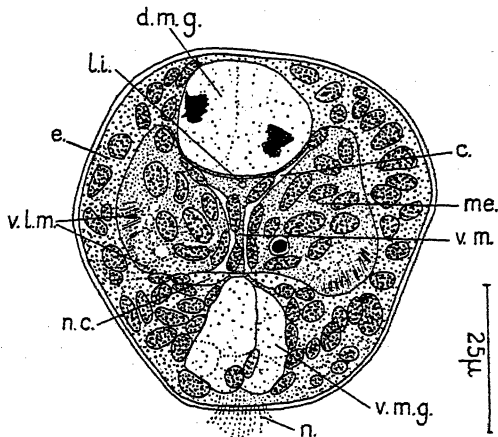
At this point it may be stated that in spite of careful search nothing resembling nephridia has been seen during the larval stages, nor have cells been distinguished resembling those in *Psygmodbranchus* referred to by Meyer as the 'vergängliche Nierenschlauchanlagen'. It is just possible that they are exceptionally minute and have been missed; but, on the other hand, no tissue has been seen that has not been satisfactorily accounted for in other ways. In this connexion one might speculate as to the possible excretory function of the large vesicles, but in the absence of proof there seems little value in so doing. After metamorphosis it is not quite so certain that no nephridia are present but nothing has been seen that can be definitely interpreted as such.

The epidermis contains a number of yolk-globules, especially in the head and particularly during the earlier stages (Text-figs. 5 and 8, *y.g.*). They become used up with increasing age. The cytoplasm is generally rather vacuolated especially in the head and the prototroch (Text-fig. 9). A very large vacuole or vesicle is generally present on each side of the mid-dorsal line immediately posterior to the prototroch; it does not, however, appear in any of the sections figured, as they do not pass through it. The prototroch shows in section the three to five bands of cilia according to position, and the ciliary rootlets are readily visible (Text-figs. 5, 8, and 9). The cuticle is thicker over the anterior part of the head than it is elsewhere; it varies in thickness over different parts of the body, and must have been added to by secretion since it formed the egg membrane.

The cerebral ganglion or brain is a large structure immediately behind the head vesicle (Text-figs. 5 and 8, *c.g.*). Fibrous-looking tissue that appears to arise from the base of the vesicle penetrates the centre of the brain, passing right through in what seems to be a minute tubular passage (Text-figs. 5 and 10, *p.c.g.*). At the base or posterior side of the brain the fibres turn outwards and run ventrally, laterally, and dorsally into the epidermis as far as the cuticle. They can be stained with Heidenhain's haematoxylin and are easily observed in transverse sections level with the base of the brain. The head epidermis is indeed penetrated in various directions by these fibres, some even reaching

anteriorly to the eye-level. It is possible that they are of a contractile nature. They disappear at metamorphosis.

In sections the brain shows up as a large clear area closely surrounded by nuclei. It appears to be composed of numerous very fine interlacing fibrils. It has not been possible to indicate these in the drawings, and the brain tissue has therefore been

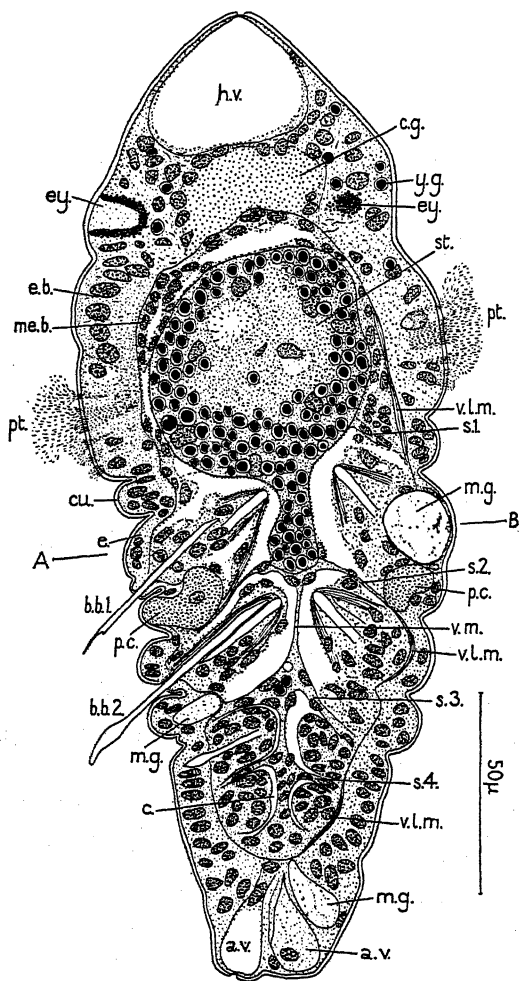


TEXT-FIG. 7.

Transverse section of posterior end of an eight-day-old larva.  
Heid. haem. Approximate plane of this section shown at  $\times x$  in  
Text-fig. 5.

conventionally dotted. The circumoesophageal nerve-cords (Text-fig. 9, *cir.n.*) can be traced round the oesophagus on each side, and they continue to the posterior end as the ventral nerve-cords, one on either side of the mid-ventral line (Text-figs. 6 and 7, *n.c.*). They show the same fibrillar structure and lie just internally to the epidermis.

The shape of the chaetae and their order of appearance will receive attention later. Their sacs doubtless arise by ectodermal inpushings that become associated with the mesoderm forming the parapodial muscles. The internal parts of the parapodia take up much of the body-cavity underneath the gut which, possibly as a result, lies towards the dorsal body-wall (Text-figs. 6 and 8).



TEXT-FIG. 8.

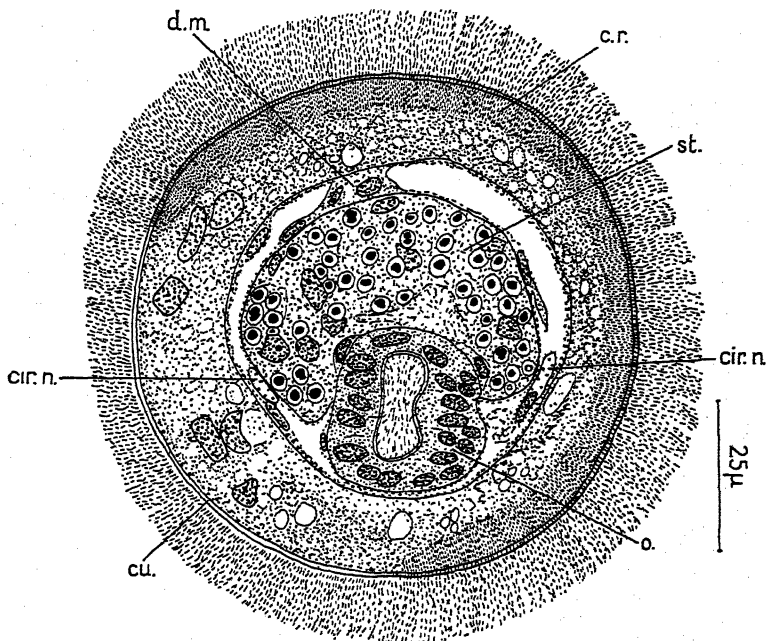
Slightly oblique frontal section of an eight-day-old larva, but a little less advanced in development than that shown in Text-fig. 5. Heid. haem. The section cuts the epidermis at a more ventral level on the right than on the left. AB. Approximate plane of section shown in Text-fig. 6.

The oesophagus is an ectodermal invagination lined internally

with cuticle and well ciliated. It is approximately circular in cross-section and has relatively thick walls (Text-figs. 5 and 9, o).

### Mesodermal Structures.

As already stated, the early origin of this and the first forma-



TEXT-FIG. 9.

Slightly oblique transverse section in the prototroch region of an eight-day-old larva. Heid. haem. On the left the section passes through the ectoderm immediately anterior to the ciliary rootlets of the first band of cilia.

tion of the segmental blocks have not been made out. When it is first distinguishable the septa and mesenteries have already been formed. The first septum encircles the gut a little way behind the prototroch (Text-fig. 8, s.1). Ventrally it joins the gut by the junction of oesophagus with stomach (Text-fig. 5, s.1) but dorsally is well behind that junction. Usually the dorsal

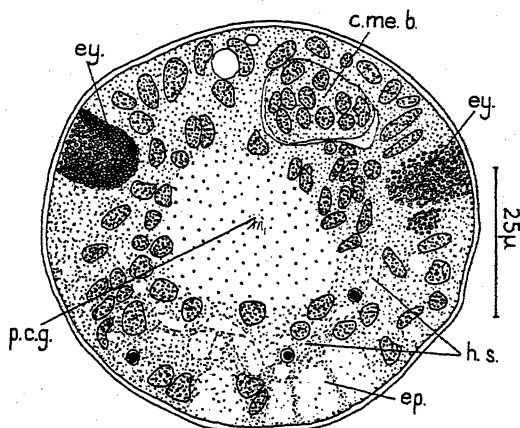


parts of the septa are indistinguishable owing to compression between the gut and the body-wall, but in a few favourable specimens they can just be made out. In late larvae there are generally three septa behind the first (Text-fig. 8). At the posterior end segmental blocks that are splitting to form coelomic cavities are visible. Anteriorly to the first septum splanchnopleuric mesoderm adheres to the gut and somatopleuric mesoderm to the body-wall, which in that region is partly collar segment and largely prototroch. The cavity in this anterior region is thus coelomic, but unfortunately it has not been possible to trace its formation, and it is uncertain whether it represents one pair of segmental blocks or more, in other words, whether or not it is homologous with the combined buccal organ and head cavity of *Owenia*. The ventral mesentery is continuous throughout. At its junction with the gut it forms the ventral blood-vessel which at this stage is triangular in cross-section (Text-fig. 6, *v.v.*). The dorsal mesentery, like the dorsal parts of the septa, is difficult to distinguish, but it can occasionally be traced running forward and passing under the prototroch (Text-fig. 9, *d.m.*) to end a short distance in front. It does not enclose a blood-vessel. The splanchnopleure is an excessively fine and occasionally nucleated membrane surrounding the gut. The somatopleure is thicker. We have already seen that it gives rise to the muscles associated with the parapodia. It is also responsible for the formation of the longitudinal muscles that in four groups, two dorsal and two ventral, run close under the epidermis from one end of the body to the other (Text-figs. 6 and 8, *d.l.m.* and *v.l.m.*). A few fine circular muscle strands are to be seen in the anterior trunk region prior to metamorphosis (Text-fig. 6, *c.m.*). In all this mesoderm cell limits are not visible.

### Endodermal Structures.

The main endodermal mass is extremely yolky throughout larval life. The yolk-globules are contained in conspicuous vacuoles in the cytoplasm. These vacuoles are represented by rings in the drawings but they do not, of course, actually have strong walls. The globules are of varying size and there are

sometimes two in the same vacuole. With Heidenhain's haematoxylin they stain very darkly, remaining unstained with Delafield's haematoxylin, when they show a certain amount of granular structure. The endodermal nuclei are often compressed into irregular shapes by these globules and their vacuoles. The



TEXT-FIG. 10.

Slightly oblique transverse section in the eye region of an eight-day-old larva. Heid. haem. The section cuts the surface farther back on the right than on the left. This larva was at an earlier stage in development than the average for its age, the branchial lobes having scarcely started to grow out.

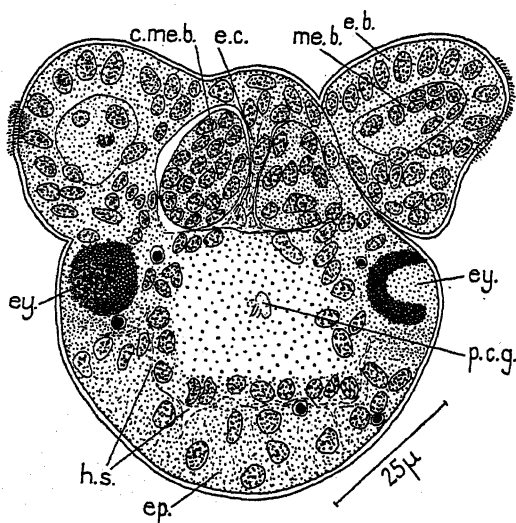
endodermal gut is at first solid, but a central ciliated lumen gradually appears as yolk is used up.

During the early stages the endoderm completely fills the body and extends right to the posterior end. Later, however, when the body is longer, the gut is posteriorly reduced to a very slender strip of endodermal tissue immediately below the dorsal mucus glands of that region (Text-figs. 5 and 7). This strip runs towards the position of the anus, which is not yet open, the lumen (*l.i.*) in it being very minute.

### The Branchial Rudiments.

The branchial rudiments arise as swellings on either side of the mid-dorsal line immediately in front of the prototroch.

They consist of rather tall ectodermal cells with relatively large nuclei (Text-figs. 8 and 11, *e.b.*). The cytoplasm is somewhat dense and cell limits can occasionally be made out. Frequent cell division takes place, the swellings enlarge and mesoderm extends up into them (Text-figs. 8 and 11, *me.b.*). This mesoderm was present as a small solid mass of fairly closely packed nuclei, immediately under the ectoderm on each side, before it



TEXT-FIG. 11.

Slightly oblique transverse section in the eye region of a twelve-day-old larva about to metamorphose. Heid. haem. This larva was also at a little earlier stage than the average for its age.

became rounded to form the branchial rudiment. Its origin is not known. It was in position when the larva was only four days old and could doubtfully be distinguished at two days, when all the tissues were very compact and very yolk. Nuclei divide in the mesoderm in pace with those of the ectoderm so that the mesodermal mass increases in size to fill the ectodermal sheath. The line of demarcation between ectoderm and mesoderm is unusually clear. The mesoderm hollows and forms the blood-vessels and other internal structures of the pinnules.

## GENERAL ACCOUNT OF THE METAMORPHOSIS.

When the larvae are about eight or nine days old and have three or four setigers marked out they settle down. In the finger-bowls they generally settle high up the sides, just below the meniscus, and are often in two main groups, one on the side nearest to the light and the other on that farthest from the light. They secrete a thin-walled tube of mucus in which they move themselves about with their bristles, the cilia of the prototroch only flickering occasionally, being obviously hampered by the tube-wall. In nature the adults live in ground that, while muddy, has a large admixture of sand and stones, and it seems very likely that the larvae first settle on pebbles lying on the surface of the substratum. A laboratory experiment designed to test this assumption gave no results.

We have stated that the larvae settle at eight or nine days with three or four setigers. There is actually wide variation in the time and stage, not only as between different cultures but in the same finger-bowl. In the latter the eggs would all have been fertilized together but came from several parents. In one instance a bowl of eggs fertilized on 23/5/33 contained larvae the majority of which settled down six days later, but some of them still swam and had not quite reached the stage shown in Text-fig. 3 nine days after fertilization. Those which had settled early did so before they reached that stage, when, in fact, they had only two setigers with bristles, the third being marked out but still bristleless. The head and pygidial regions began to metamorphose before the body was as fully developed as in Text-fig. 3. One very precocious larva, trapped in a cavity slide when only three days old and very similar in appearance to Text-fig. 2, secreted a thin-walled mucus tube at the edge of the cavity between the slide and the cover glass. It remained in the tube for several hours, leaving it overnight probably owing to lack of aeration of the water in the cavity, for it was not possible to move the cover glass without damaging the tube. The larva had become unhealthy, as they always do when left long in a sealed and confined space. If this fairly wide range of settling time occurs in nature it may increase the chances of

the larvae arising from any particular act of spawning ultimately reaching the right ground. It is possible, too, that a larva can metamorphose at any time over a period of several days and does so as soon as it strikes a favourable bottom. It has not, however, been possible to prove this, as has been previously done for *Owenia* (Wilson, 1932) and *Scolecolepis* (Day and Wilson, 1934), for the sides of any containing vessel are obviously suitable places for a species that settles on a hard surface.

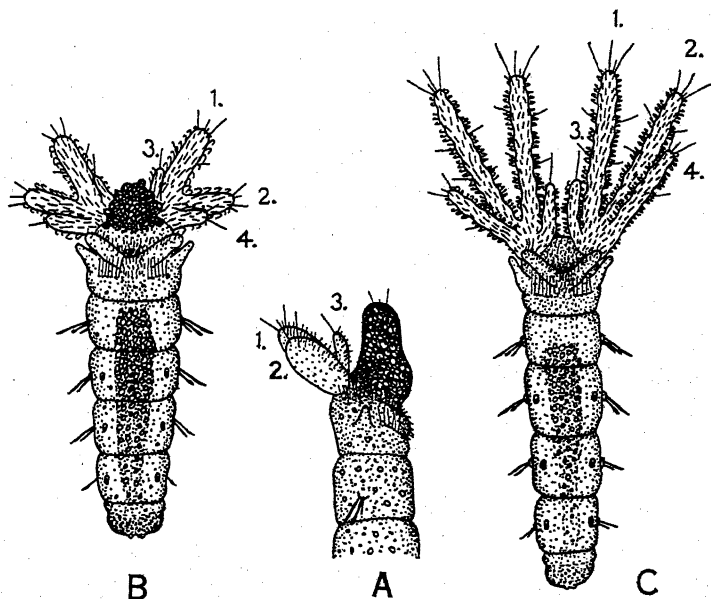
Metamorphosis is a gradual process, spread over several days, while the larva lives in its newly formed tube. The cilia of the prototroch become unable to beat properly if the larva be removed from its tube. A mid-dorsal gap appears in the prototroch and gradually widens until finally all the cilia have disappeared, whether thrown off or absorbed has not been determined. The prototroch cells apparently move ventralwards from the dorsal parts. Meanwhile the head vesicle shrinks and most of the head tissue ventral and anterior to the insertion of the branchial rudiments and the eyes, with the addition of the prototroch cells, slowly breaks down into a yellowish-brown granular-looking mass, that forms a snout-like protuberance between the branchial rudiments and the mouth (Text-fig. 12 A). The eyes themselves begin to break down and give off about half their pigment globules, which migrate singly and in clusters into the snout. In this way the eyes become shallow instead of deep cups and remain as such throughout the further changes.

During formation of the snout the branchial rudiments have been growing and branching rapidly. There are now three lobes to each, two large almost equal ones and a shorter slenderer one situated a little in front of the others and next to the middle line (Text-fig. 12 A). These branches are all ciliated ventrally and carry a number of sensory cilia.

The pygidial region loses its vesicles and acquires brown pigment specks. The anus opens on its dorsal surface and becomes ciliated.

The neurotroch disappears with the exception of a patch of cilia immediately posterior to the mouth. These may possibly be new cilia in place of those previously forming the anterior

end of the neurotroch. A short line of a few long cilia appears on either side of the old neurotrochal groove, roughly between it and the growing collar lobe. A fold of tissue arises on either side of the mouth and becomes ciliated. These folds are the



TEXT-FIG. 12.

Metamorphosis of *Branchiomma vesiculosum*. Three individuals at different stages removed from their tubes and drawn from life.  $\times 156$ . The numbers opposite the branchial pinnules refer to the order of their appearance. A. Nine days old. View of right side. Snout fully formed but not yet breaking up. B. Ten days old. Ventral view. Snout breaking up. About half of it has already disappeared. C. Twenty days old. Ventral view. A few days after completion of metamorphosis. Actual length, tip of longest branchial pinnule to posterior extremity, approx.  $440\mu$ .

ventral lips; they meet one another in the mid-ventral line, forming a V.

When the granular snout has been fully formed, covered, of course, by the thick cuticle that everywhere envelops the body, it begins to break up. Little pieces of tissue every now and

again come off the main mass and float away. The snout becomes very irregular (Text-fig. 12 B) with little rounded lumps of tissue sticking out of it everywhere. These lumps are pieces almost ready to break off. Slowly the size of the mass is reduced and in about two days it has entirely disappeared (Text-fig. 12 C) leaving no trace. Metamorphosis is now complete. As the snout itself took about two days to form before it began to disintegrate, the total time taken to metamorphose is about four days.

The worm begins to feed just before or during metamorphosis, and its stomach at that time often contains a brownish mass of food. Diatom frustules are also occasionally seen in sections of the gut. This brownish mass of food has frequently been observed before the snout started to be formed, let alone break into pieces. It is therefore not necessarily discarded larval tissue that has been swallowed. On the other hand, it seems unlikely that some of the pieces of disintegrated snout are not swallowed, and yet it is very difficult to produce definite evidence that they are. Sections of metamorphosing larvae frequently show granular matter in the gut, that compares closely with that of the broken down head tissue, and in a few instances globules resembling those cast off from the eyes are seen. It is hard to resist the conclusion that this is swallowed larval tissue, although it may have come from other individuals close by. I have spent many hours watching metamorphosing larvae, but, except in one doubtful instance, never saw a piece swallowed. On the other hand, I have seen on more than one occasion snout tissue break off and, caught by the cilia of the pinnules, be drawn down to the mouth there to whirl round and round and even travel some way down the oesophagus. Again and again the piece would be expelled, only to be attracted back to the mouth, carried there by the ciliary currents, slowly breaking into smaller and smaller pieces, some of which drifted away to be finally lost. The worms could quite well have swallowed the pieces had they so wished. Their reactions may have been affected by the discomfort of the strong light and the cavity slides in which they were confined. Possibly under more natural conditions they do swallow some of their own tissue, but I think

it unlikely that they eat all the snout. At any rate, the quantity of food matter in the stomach at any one time is only a small proportion of the total amount of snout and some of it consists definitely of diatoms. Unless the tissue is digested soon after it is swallowed, the stomach never contains sufficient matter to account for all the larval head tissue that disappears.

While the broken down larval tissue is being got rid of, other changes are taking place in the head. The mouth has enlarged from the small opening that it was prior to metamorphosis, and the disappearance of the snout causes it to be directed forwards almost as a funnel at the bases of the branchiae. The latter continue to grow and by the time metamorphosis is complete have each four fairly long lobes or pinnules (Text-fig. 12 c). Their bases have been twisted forwards and somewhat downwards and have grown over the remnants of the eyes so that the latter are now situated deep in the head behind the roots of the branchiae.

#### DETAILED STUDY OF THE METAMORPHOSIS.

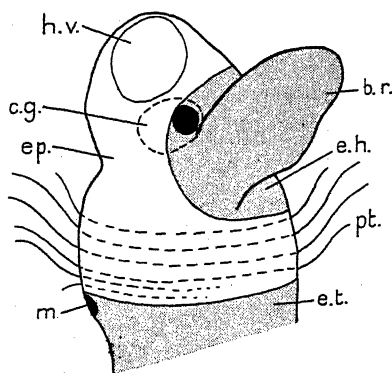
Before it is possible to appreciate the significance of the curious metamorphosis just described it is necessary to be fully conversant with the structure of the larval head before it begins. Some details have already been given, but those that have a special bearing on the metamorphosis have been left to this section.

A close study of sagittal and transverse sections of the late larval stages reveals an ill-defined irregular split under the brain between it and the ventral ectoderm of the head (Text-figs. 5, 10, and 11, *h.s.*). The split runs up on either side almost to the level of the eyes. It is difficult to see but can be traced in all good sections, in some better than in others. It is probably crossed by protoplasmic filaments from the tissues on either side.

The tissue that breaks down to form the snout and is ultimately cast off consists, in addition to the prototroch, of this ventral head ectoderm (*e.p.*), the head vesicles, and tissue dorso-anterior to the brain as far as some ill-defined point above it. The rest of the head region—roughly the posterior dorsal part bordered by an undefined line running close by the prototroch,



turning sharply forwards below the base of each branchial rudiment and rounding the eye to turn sharply upwards towards the mid-dorsal region—consists of a relatively small saddle-shaped region that ultimately forms the adult head (Text-fig. 13). There is no clearly marked distinction in the larval stage between adult and larval head tissues as there is in *Polygordius* and *Owenia*, only the different fates of the two

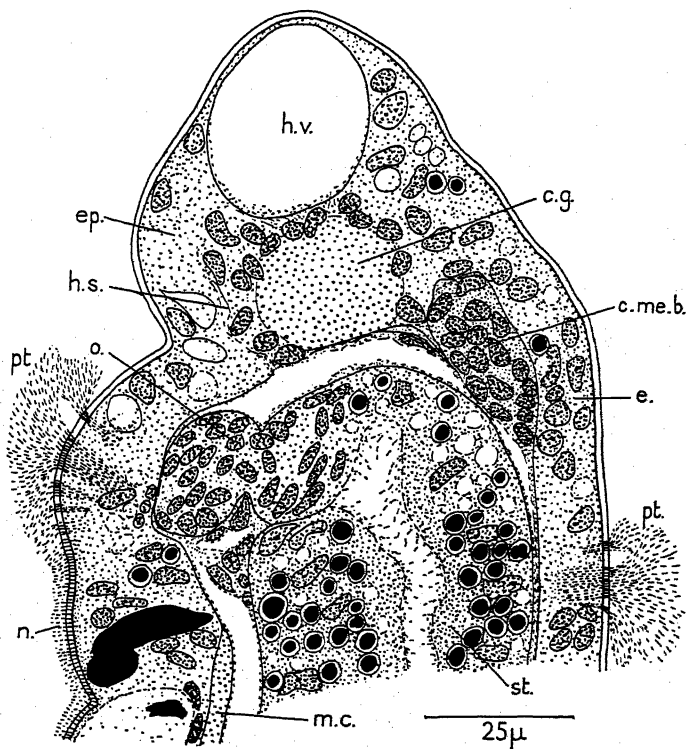


TEXT-FIG. 13.

Diagram of the head region of an advanced larva showing approximately the relations between the larval ectoderm (unstippled) lost at metamorphosis and the adult ectoderm (stippled).

tissues at metamorphosis serve to distinguish them. It seems clear, however, that in spite of the great difference in general external appearance of the metamorphosis of *Branchiomma* compared with that of *Polygordius* and *Owenia*, the process is fundamentally the same. In the two latter species the adult head is separated from the prototroch by a wide area of thin episphere tissue all round it, in *Branchiomma* the head is practically in contact with the prototroch dorsally, but laterally, and especially ventrally, is separated from it by a well developed and very thick wall of episphere cells (Text-figs. 5, 10, 11, 13, and 14, *ep.*). The head vesicle is presumably simply one of these cells specially developed and highly vacuolated. It is roughly in the same morphological position as the large 'refringent sphere' that is invariably present in the episphere of

the *Mitraria* larva a little distance in front of the head. The brain, however, instead of being situated immediately below the ectoderm of the head is shifted forward a little and tends to fuse temporarily with the episphere tissue. Later, as we shall



TEXT-FIG. 14.

Sagittal section of head end of an advanced larva cut a little to one side of the median plane. Heid. haem. This section is from the same larva as that shown in Text-fig. 5.

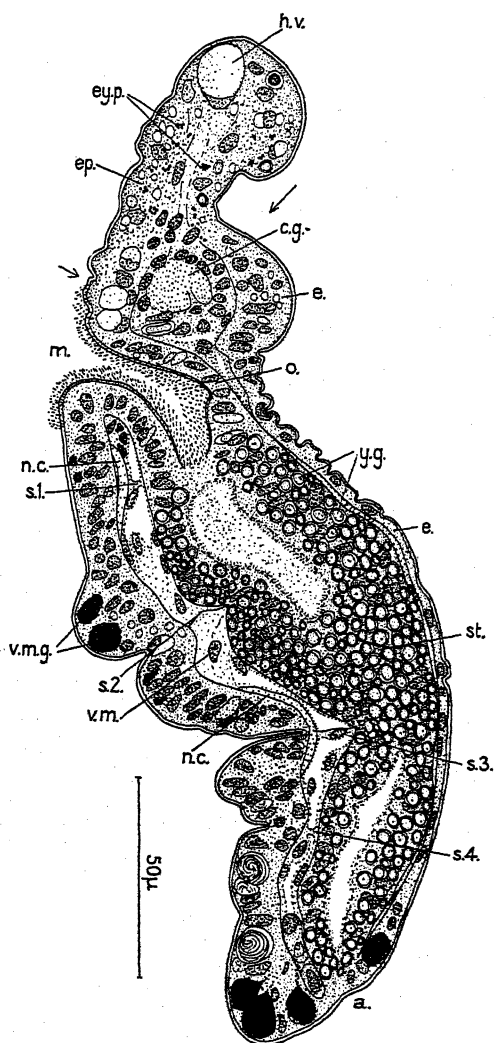
see, it shifts back in relation to the adult head ectoderm, which ultimately caps it over.

On either side between the mid-dorsal line and the base of the growing branchial rudiment there is to be seen a mass of

mesoderm (Text-figs. 10, 11, and 14, *c.me.b.*) that laterally is connected with the mesoderm inside the branchial rudiment itself. A little before and during the early stages of metamorphosis these two masses extend forwards some way above the brain and cut it off from the ectoderm dorsal to it, although the brain may still be so connected in the mid-dorsal line for a time (Text-fig. 11, *e.c.*). This connexion disappears during early metamorphosis, with the result that the brain tissue is cut off from direct connexion with the adult head ectoderm except in the region of the eyes.

The further history of the mesoderm masses is difficult to follow, but it seems certain that they take part in the formation of the strong, dorsal, longitudinal muscles running above the eyes to be inserted on the branchial skeleton (Text-figs. 20, 21, and 25, *d.l.m.*).

The prototroch begins to disappear at the mid-dorsal line. The prototroch 'cells' in this region shorten so that the prototrochal ring becomes thinner there than elsewhere. This process has already taken place in the larva drawn in Text-fig. 5, and the thickness of the prototroch dorsally is only little more than half what it was and what the ventral portion still is. When thinning to about this degree has occurred cilia disappear dorsally, and the actual cell tissue of the prototroch is apparently pushed to either side as the head ectoderm joins on to the body ectoderm in the mid-dorsal line. It must be understood that the details of this process are by no means clear, for the absence of cell walls and sharp lines of demarcation between the prototroch and other tissues make it very difficult to follow, and one can only surmise what is happening from the general appearances of large numbers of individuals. The dorsal gap in the prototroch widens, the cilia presumably either falling off or being absorbed, or a combination of both processes may take place, as Day (1934) has described for the larger cilia of *Scolecoplepis*. The prototroch cell tissue is worked gradually downwards behind the branchiae as more and more of the head joins on to the trunk. Finally it is worked low enough for it to be incorporated in the posterior ventral part of the snout. At any rate, there is no evidence of internal absorption taking place. The

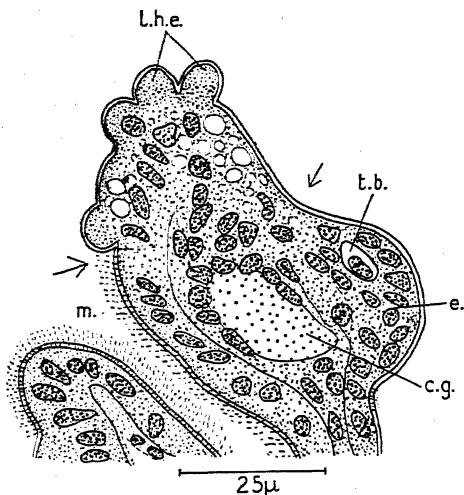


TEXT-FIG. 15.

Median sagittal section of a metamorphosing larva eight days old. Del. haem. The body of the specimen was not perfectly straight, so that in two places one of the nerve-cords appears in the section. Arrows indicate approximately the places where adult and larval ectoderms are in fusion.

ventrally placed cilia are the last to be lost; by the time the snout begins to break up they have usually disappeared.

The formation of the snout is a gradual process. The head vesicle shrinks and is carried forwards away from the brain (Text-fig. 15), and much of the episphere tissue (*ep.*) that lay ventral and posterior to the brain is carried in front. The brain



TEXT-FIG. 16.

Median sagittal section of head of a metamorphosing larva in which more than half of the snout had already been cast off, and other pieces are rounding up to break away. Del. haem. Arrows as in preceding figure.

loses its connexions with the larval episphere tissues and begins to move back relative to the adult head and body tissues. The central perforation persists for a time at least, and serves to show that the brain is not rotated on the body axis during this process. It, however, shrinks to some extent.

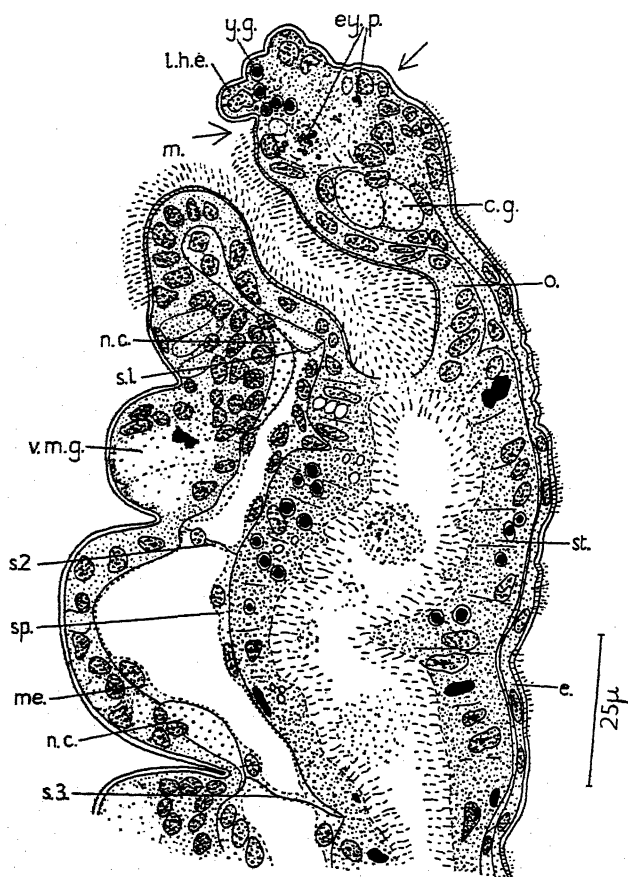
In sections the fully formed snout, that in the living animal is conspicuous by reason of its granular and yellow-brown appearance, shows no very special features, except for the absence of mucus cells in the ventral episphere, for they discharged their contents at the onset of metamorphosis, and in so

doing probably assisted the mucus cells of the trunk in the formation of the larval tube. The snout appears to be highly vacuolated and spongy, but not very much more so than the head tissues in earlier stages. Its nuclei look normal and do not break down. Varying quantities of yolk-globules are present and do not seem to disintegrate even when rounded up into pieces for breaking away (Text-fig. 17, *y.g.*). The reddish pigment globules discarded from the eyes are the most conspicuous strange feature, they scatter singly and in clumps throughout the snout (Text-figs. 15 and 17, *ey.p.*), a fact that seems to indicate a certain state of fluidity for those tissues. The cuticle maintains a close adhesion to the snout, following closely every little irregularity and even completely surrounding each little particle as it is rounded off for discharge (Text-figs. 16 and 17), and finally forming a coat to each separated particle.

The loss of the prototroch resulted in the posterior mid-dorsal border of the head becoming continuous with the dorsal body ectoderm. Similarly the final disappearance of the snout brings the anterior border of the head into contact and fusion with the dorsal anterior end of the oesophagus (cf. Text-figs. 15 and 18), the ventral end of the latter being of course continuous all along with the ventral body ectoderm. The sides of the head become at the same time continuous with the body ectoderm. This again is fundamentally the same as the process that takes place so rapidly in the cataclysmically metamorphosing *Owenia*.

Before metamorphosis the first septum ventrally joins the gut at the junction of oesophagus and stomach, and runs up round the widest part of the stomach to join the dorsal body-wall behind the prototroch. After metamorphosis it surrounds the junction of oesophagus with stomach showing that the dorsal part of the latter has been pulled back through the septum, sliding presumably inside the splanchnopleure. The same thing occurs during the metamorphosis of *Owenia*. In later development the oesophagus itself is carried back some way behind the septum, and in Text-fig. 26 it is seen that its junction with the stomach is near the middle of the first setiger.

During the progress of the above changes the branchiae have



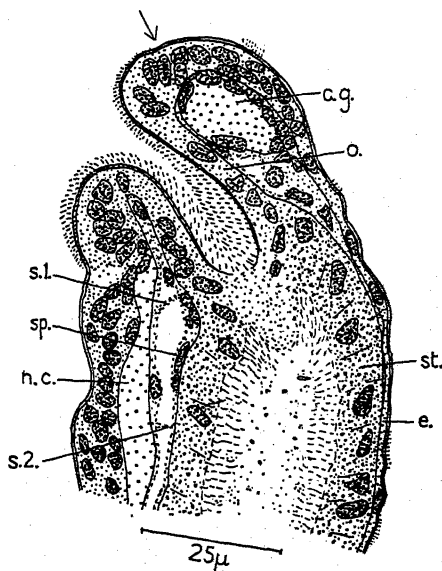
TEXT-FIG. 17.

Median sagittal section of a larva towards the end of metamorphosis.

Heid. haem. The specimen was not quite straight so that portions of one of the nerve-cords are seen. Arrows as in Text-figs. 15 and 16.

been steadily growing larger and branching. At first they were merely lobes just behind and rather above the eyes (Text-fig. 3), but as they grew their basal attachments to the head increased in width and extended downwards to below the level of the eyes. These basal parts now grow forwards over the remnants

of the larval eyes until the latter are entirely covered and appear well behind the place at which each lobe branches into the pinnules. The result is that the eye peers through two layers of ectoderm (the outer and inner walls of the branchial lobes) and the thin layer of mesoderm and possibly coelomic cavity that at the level of the eyes separates these two ectodermal



TEXT-FIG. 18.

Approximately median sagittal section of head end of young worm in which metamorphosis is complete. Heid. haem. Arrow shows approximate place of fusion of antero-dorsal extremity of oesophagus with anterior border of adult head ectoderm.

walls (Text-figs. 19 and 20). The cuticle of the inner side of the branchial processes comes into contact and apparently fuses with that covering the eyes and the head ectoderm in their immediate neighbourhood. This layer of cuticle sandwiched between head and branchial processes is clearly traceable forwards to the anterior end of the head (Text-fig. 19, *cu.*). In this arrangement we have evidently the first stage in the



development of the dorsal pit and its connexions described for *Sabella* by Nicol (1930, pp. 542-3).

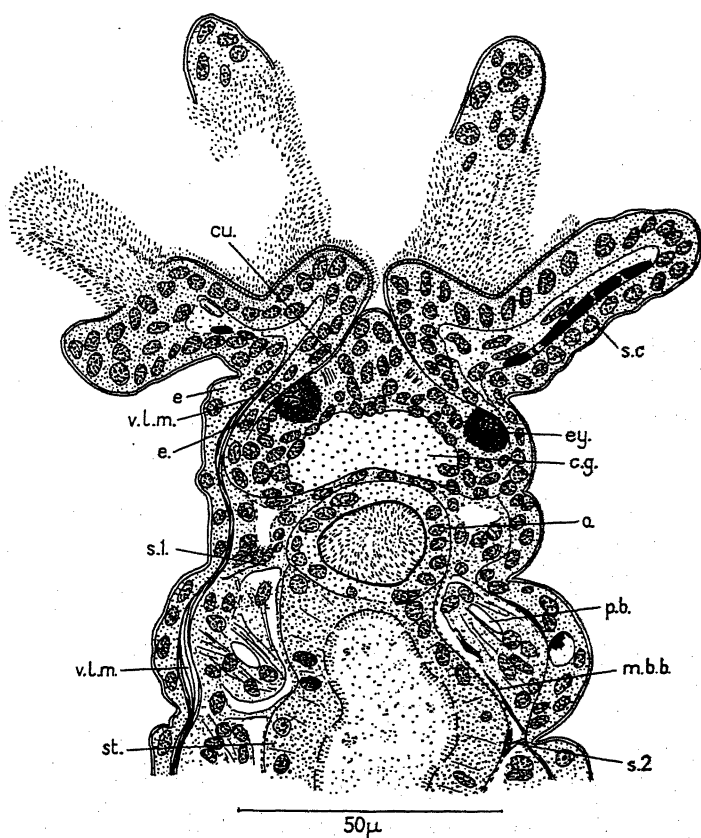
Skeletal supporting cells appear in the pinnules at an early stage. Lying end to end, they form a rod in each pinnule and near their bases run into a transverse bar that, passing through the dorsal anterior border of the head ectoderm, connects the branchial skeleton of either side. The stage in metamorphosis at which this bar can first be distinguished varies, but it is often visible before the larval snout has gone, and then serves the useful purpose of marking the position of the anteriormost part of the adult head ectoderm (Text-fig. 16, *t.b.*).

By about the end of metamorphosis two main bundles of muscle-fibres can be seen on each side, running from the body-wall to the branchial lobe, one passing above the eye and one below (Text-fig. 21, *d.l.m.* and *v.l.m.*). The formation of the dorsal muscles has already been referred to. Both dorsal and ventral muscles are really anterior prolongations into the head of the powerful dorsal and ventral longitudinal muscle-bundles that run down the trunk on either side. Their anterior ends are inserted on the base of the branchial skeleton (Text-fig. 25).

The ventral lips, that arise during metamorphosis as ciliated ectodermal ridges, are flush with the general body surface in the mid-ventral line, but become more pronounced towards their anterior lateral extremities, where they project as prominent rounded corners that come into close contact—but do not fuse—with the basal parts of the most ventral filaments of either side (Text-figs. 12 c, 22). Later the basal part of pinnule 4a (see below) becomes somewhat enlarged and swollen (Text-fig. 23, *b.4a.*), making close contact with the lips (*l.*) on each side, although there is never any actual fusion.

The collar lobes grow longer; they are situated in front of the first septum. Their structure is too indefinite for description, but they are probably sheaths of ectoderm filled with mesodermal tissue. Apart from vacuoles in the cytoplasm they seem to be solid structures without cell walls. They are not ciliated.

When metamorphosis begins a great deal of yolk still remains in the walls of the gut, the lumen of which is really only just beginning to open up and the cells bordering it to become

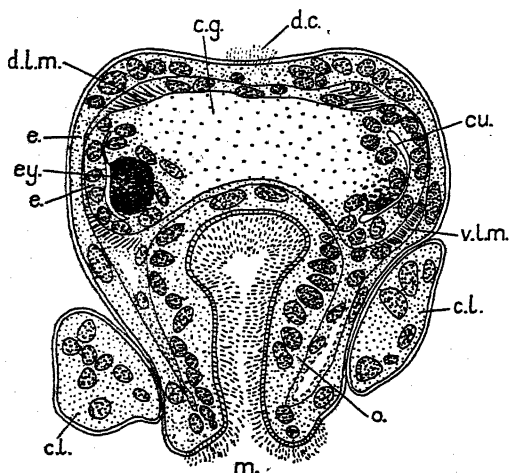


TEXT-FIG. 19.

Slightly oblique frontal section of anterior end of a young worm in which metamorphosis is complete. Heid. haem. The section cuts the ectoderm on the right slightly nearer to the dorsal surface than on the left. In the original section the relations of the tissues near the left eye were clear, but owing to the angle of cut they were not so clear on the right.

ciliated. During metamorphosis the yolk-globules that stain so darkly with Heidenhain's haematoxylin are rapidly reduced in number, apparently being absorbed. By the time that metamorphosis is complete few or none of these globules remain (cf. Text-figs. 5, 15, 17, and 18). On the other hand, some very

small spheres that can be seen in the cytoplasm of the gut cells from quite early stages become relatively prominent. They stain only lightly with Heidenhain's haematoxylin, but during and after metamorphosis become very dark with Delafield's haematoxylin. These changes in the appearance of the gut walls can be regarded as indicative of changing functional activity, conse-



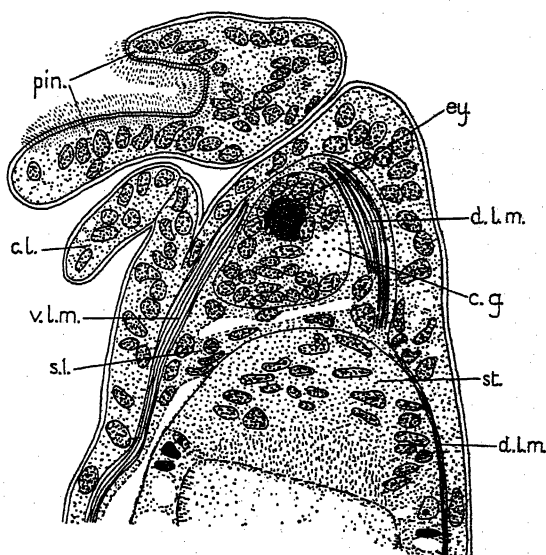
TEXT-FIG. 20.

Slightly oblique transverse section of the eye region of the head of a fully metamorphosed young worm. Del. haem. The section cuts the ectoderm immediately behind the eye on the right. A few pigment globules belonging to this eye are visible.

quent upon preparations for the digestion and absorption of food taken in through the mouth. The anus opens during early metamorphosis and occupies a dorsal position on the pygidium. A slight ciliated ectodermal invagination forms a short proctodaeum (Text-fig. 27, p.).

The body-wall of the ventral shield-gland region of the metamorphosing worm becomes much thinner than it was during the larval stage (cf. Text-figs. 5, 15, 17, and 18). This is doubtless due in part at least to the discharge of mucus from the gland-cells that mainly compose that region. The mucus is used to form and thicken the tube wall. Later on, after meta-

morphosis, this shield-gland area becomes thicker again, with large well-filled gland-cells (Text-fig. 26, *v.m.g.*). The thinning of the wall may also be due in part to an actual elongation lengthwise of the tissues composing it. Such an elongation would allow the worm greater latitude in stretching and con-



TEXT-FIG. 21.

Lateral sagittal section through eye of a fully metamorphosed young worm. Heid. haem.

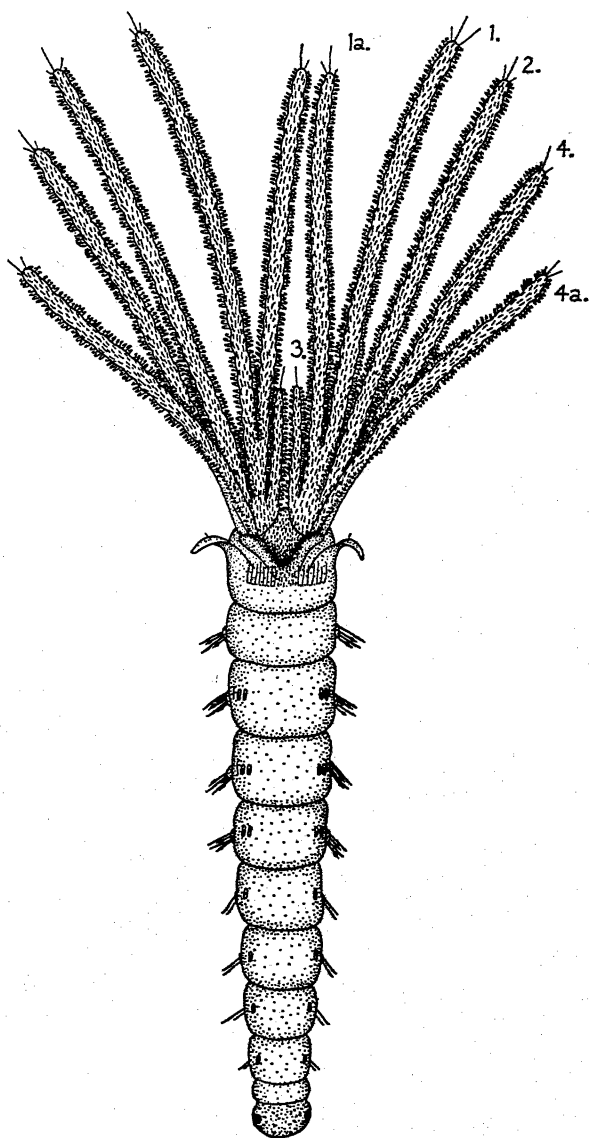
tracting. The ridge-like ectodermal thickenings of the dorsal and lateral body-walls (in fixed specimens) also become thinner at the same time and possibly for the same reason. The dorsal mucus cells, which in the larva are situated anterior to the future position of the anus, discharge their contents during metamorphosis and disappear.

#### JUVENILE STAGES.

After metamorphosis the main changes that take place during the juvenile stages, as far as reared, are confined largely to an

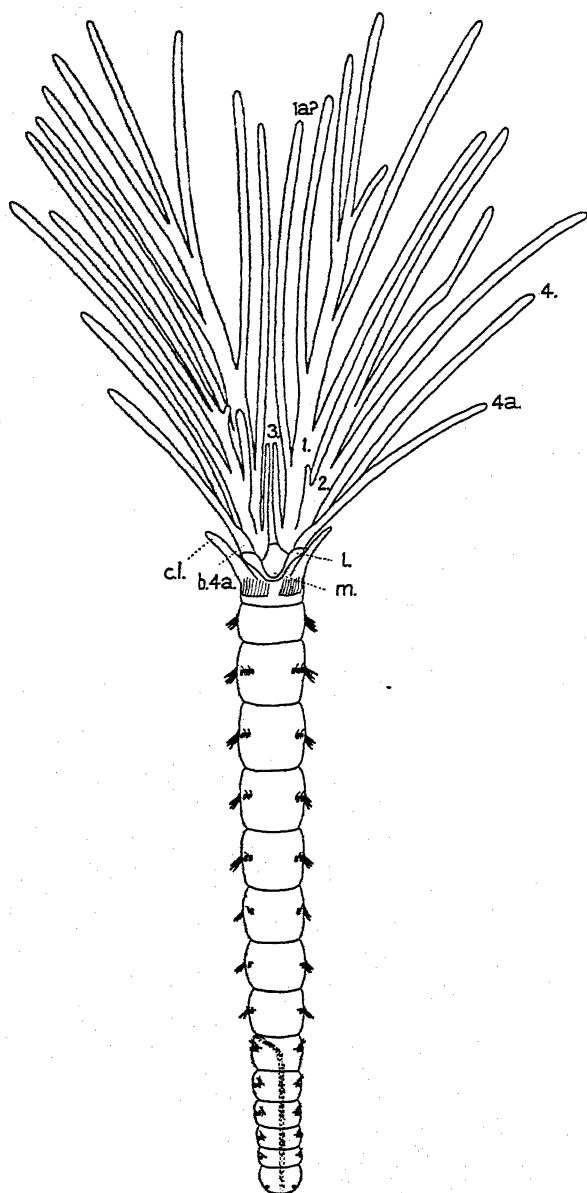
increase in complexity of the branchial crown and increasing segmentation with elongation of the trunk. It might be as well to begin our account of the branching of the branchial rudiments at the stage when they first appear during larval life. As already mentioned, they arise as a pair of large prominences on either side of the head anterior to the prototroch. It is not long before each shows a slight elevation on its posterior side (Text-fig. 3) that rapidly grows until the lobe appears to be bifurcated. About the same time a third and smaller bud appears on the inside or ventral anterior part of the lobe. Thus for a short time during early metamorphosis each branchial process shows three short branches of unequal length (Text-fig. 12 A). Soon a fourth branch develops near the base of the second one (Text-fig. 12 B), so that when metamorphosis is complete four primary branches are present. Throughout the succeeding stages the third of these remains short and ultimately forms the so-called palps of the adult. The other three branches, which show the structure of pinnules, grow steadily longer and themselves bud off pinnule-like branches. One of these (Text-fig. 22, 1a) arises near the base and on the inner side of the first and longest branch of all, and the other (Text-fig. 22, 4a) near the base and on the outer side of the fourth pinnule. Both grow quickly until they are almost as long as their parent branches. The fourth branch does not bud again as far as the young worms have been reared, but the first and second each give rise to a number of pinnules. In these later stages budding may not be quite even on both sides (Text-fig. 23).

The branchial rudiments become ciliated ventrally soon after their first appearance, and after metamorphosis the individual branches acquire the type of ciliary arrangement characteristic of adult pinnules. A transverse section of a pinnule about the stage of Text-fig. 22 is shown in Text-fig. 24. It will be seen that the ventral surface carries numerous short frontal cilia (*f.c.*) and at each side longer latero-frontal ones (*l.f.c.*). Dorsally there is a narrow band of abfrontal cilia (*ab.c.*); these do not extend right to the base of the pinnule but stop a short distance above it (Text-fig. 25, *ab.c.*). They beat distally in a direction opposite to that of the ventral tract. The latero-frontal cilia



TEXT-FIG. 22.

Young *Branchiomma vesiculosum* fifty-two days old removed from its tube and drawn from life.  $\times 130$ . Actual length including branchial pinnules approx.  $1,140\mu$ . The numbers opposite the branchial pinnules refer to the order of their appearance.



TEXT-FIG. 23.

during life curve inwards towards the ventral surface; they are arranged in short discontinuous rows on each side of the pinnule, which is slightly annulated. The frontal cilia beat downwards towards the mouth. Nicol (1930) states that these cilia in the adult *Branchiomma* function in exactly the same way as those she has carefully described for *Sabella*. Sensory cilia are also present on the dorsal surfaces of the pinnules.

Internally the pinnules are supported by the so-called cartilaginous or skeletal cells. These have very thick walls and a large nucleus; with the exception of the basal cells they are all elongated and placed end to end along the length of the pinnule, becoming longer and somewhat narrower the more distally they are situated (Text-fig. 25). The main longitudinal dorsal and ventral muscle-bundles are attached to one of the basal cells on each side (Text-fig. 25). As previously mentioned, a transverse connecting bar crosses the snout (Text-fig. 26, *t.b.*) in front of the eyes and unites the branchial skeletons of either side. In transverse sections there may be seen, immediately central to the skeletal cells, a blood-vessel (Text-fig. 24, *b.v.*). Gland-cells can occasionally be distinguished in the epithelium of the pinnules, which are clothed like the rest of the body in a fairly thick cuticle.

The third branchial branches or palps differ markedly from the others, for they possess no supporting skeleton. This

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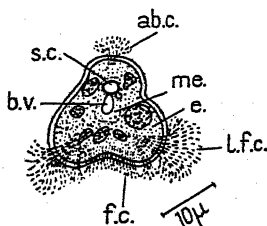
TEXT-FIG. 23 (*contd.*).

Young *Branchiomma vesiculosum* fifty-eight days old removed from its tube and drawn from life.  $\times 75$ . Actual length including branchiae approx. 2.1 mm. The numbers opposite the branchial pinnules refer to the order of their appearance. In comparing this specimen with that shown in Text-fig. 22 it may be noted that whereas the latter was reared from mid-June to mid-August 1929, with an average daily air temperature of  $65.8^{\circ}\text{F.}$ , the former was reared from mid-July to mid-September 1929, with an average daily air temperature of  $67.3^{\circ}\text{F.}$  as calculated from the routine laboratory readings taken twice daily. The temperature of the water in the bowls in which they were kept would follow closely that of the air. This slightly higher temperature, the little longer rearing period, and probably individual variation in the growth-rate due to differences in the amount of food obtained or other causes, readily account for the apparently rapid growth during six days.



absence definitely proves that these branches are the so-called palps, for Brunotte (1888) has pointed out that they are so characterized in the adult. In *Sabella* the branchial skeleton extends into the palps.

While the branchiae have been growing the trunk has been elongating by the addition of segments at its posterior end, immediately in front of the pygidium. At the same time it



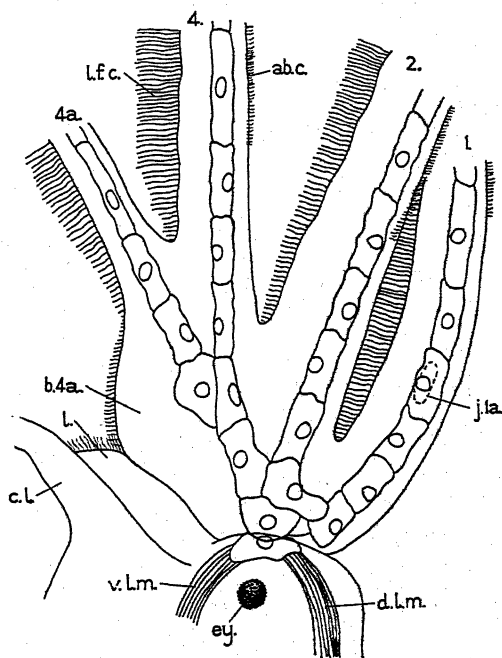
TEXT-FIG. 24.

Transverse section of distal half of a pinnule of a young worm at about the stage shown in Text-fig. 22. Heid. haem.

gains in girth. The segments added are at first setigers of the thoracic type with dorsal bristles and ventral uncini. When eight of these (including the first setiger which lacks uncini) have been formed (Text-fig. 22) the constitution of the following ones is changed and the ninth and succeeding setigers are of the abdominal type, with dorsal uncini and ventral bristles from the first (Text-fig. 23). Thus during normal development there is no change over of anterior segments from thoracic to abdominal constitution.

Apart from the cilia in the mouth region the young worm after metamorphosis has no cilia on its ventral surface. On the other hand, the dorsal surface of the trunk, which was devoid of cilia during larval life, is now ciliated along its whole length forward from the anus (Text-figs. 26-8, *d.c.*), and this line of cilia—the dorsal part of the adult faecal groove—is continued forward as far as the anterior end of the head. When the ninth setiger forms, the intersegmental groove between it and the eighth becomes ciliated on both sides. Several specimens with ten setigers, examined carefully when alive, have shown that

both the dorsal and the ventral surfaces of the ninth and tenth setigers are ciliated, the dorsal cilia being the stronger and more easily visible. The pygidium at this stage is also ciliated dorsally, but doubtfully so on the ventral surface. This posterior ciliation is the opposite of what might have been expected, for

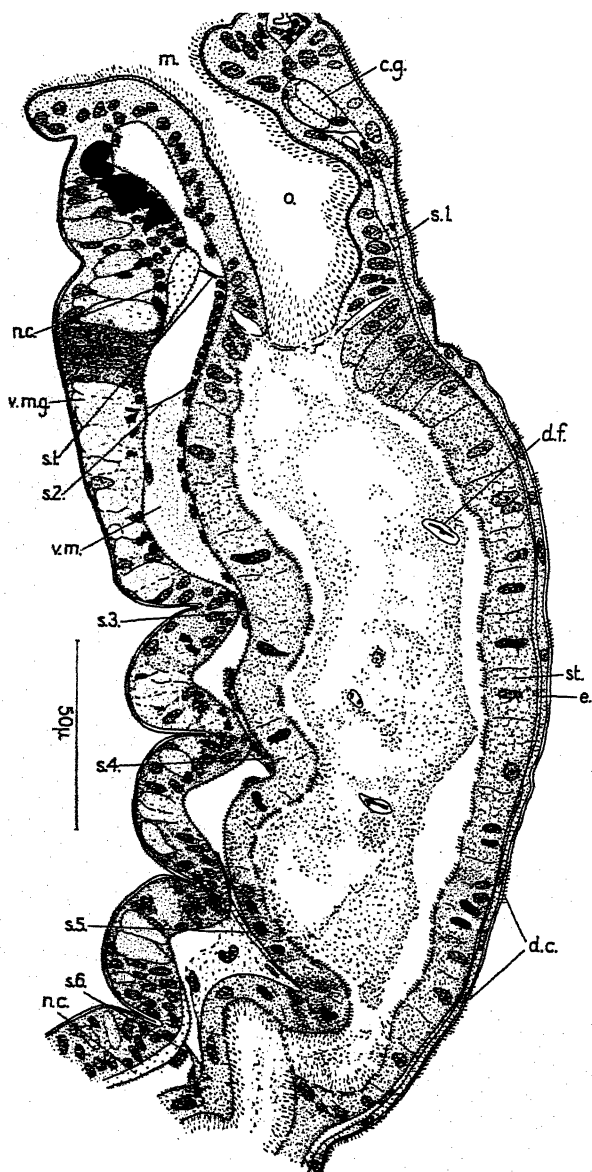


TEXT-FIG. 25.

Lateral view of arrangement of branchial skeleton of the young worm shown in Text-fig. 22. Drawn somewhat diagrammatically from the fixed specimen mounted unstained in Euparal.

in the adult it is the mid-ventral surface of the abdomen that is strongly ciliated, forming the faecal groove.

Stages later than about ten setigers were obtained once only during one of the first rearings. They were few in number and were not examined too closely in case they should be damaged, for it was hoped to rear them much further. That drawn in Text-fig. 23 was one of the oldest obtained and was fixed after



TEXT-FIG. 26.

Median sagittal section of a young worm at the stage of that shown in Text-fig. 22. Heid. haem. The worm has fixed nearly but not quite straight, so that portions of one of the lateral nerve-cords appear in two places.

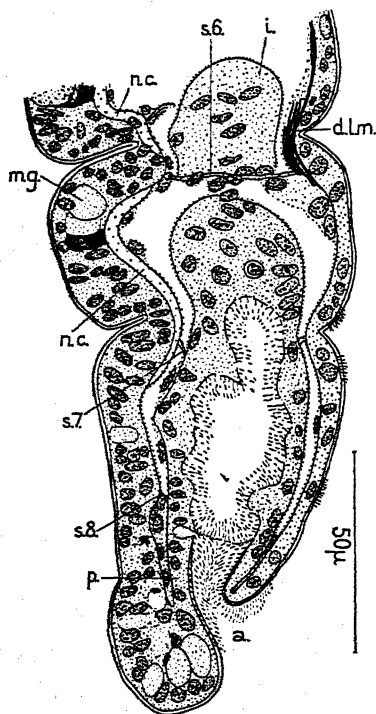
the drawing had been made. Unfortunately, at the time no attention was paid to the ciliation of the posterior segments, but examination of the mounted specimen some years afterwards shows that the ventral surface of the abdominal segments are strongly ciliated, and that this line of cilia passes to the right between setigers eight and nine, in approximately the position indicated. No cilia have been detected in the same intersegmental groove on the left side of the body, and none on the dorsal surface of the abdominal segments.

The pygidium of these young worms is speckled with brown pigment. The anus opens on it dorsally (Text-fig. 27), and it carries on either side a brown eye-spot, composed of clustered globules (Text-figs. 22 and 23), that is often more or less cup- or ring-shaped. The pygidial eyes vary in size and are sometimes irregular or absent; they first appear when five or six setigers have been formed. Probably they are of some use to the worm when it is crawling backwards tail first, with the branchial pinnules pressed together as they trail behind. It crawls readily thus if removed from its tube, and after a time settles down and secretes another mucus tube round itself. In nature it perhaps occasionally leaves its tube to seek a more favourable situation.

By the time that all thoracic setigers are present a pair of otocysts can be seen clearly. A little swelling at the dorso-anterior base of the first bristle tuft is seen to be hollow, and close examination of sections shows that it communicates with the exterior by a minute passage opening at the anterior base of the swelling, practically into the intersegmental groove marking the anterior border of the first setiger. The opening is ciliated and the cilia can be watched beating during life. The otocysts generally appear to be empty but they may contain a few specks of matter. Fauvel (1904) has described the structure of the otocyst in the adult and shown how the canal leading from it to the exterior opens inside the collar '*dans l'espèce de rigole circulaire que celle-ci fait avec la paroi du corps*' (p. 362). In the latest stages to which the young worms were reared the collar is still a long pointed lobe on either side, arising in front of and below the first bristle bundle. This, evidently the ventral element of the collar, must later extend upward, and

some relative adjustments be effected in order that the opening of the otocyst shall come in front of it.

With regard to the internal tissues there is little of special

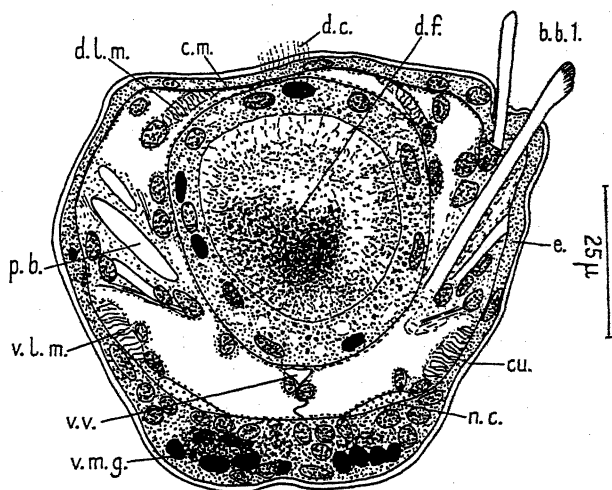


TEXT-FIG. 27.

The posterior extremity of the worm shown in Text-fig. 26, taken from the same series. As the tail was bent a little to one side it is not a continuation of the same actual section but of the third section to one side.

interest to record. The circular muscles, which first appeared in the anterior setigers before metamorphosis as a few thin fibres, developed more especially in the dorso-lateral parts of the body-wall between the longitudinal muscles and the epidermis, increase slowly in number and strengthen after metamorphosis. Yet at the latest stages they are relatively few and

unimportant (Text-fig. 28, *c.m.*). The longitudinal muscles, however, form four large powerful bundles that run from one end of the body to the other. They are arranged as dorsal and ventral bundles on each side (Text-fig. 28, *d.l.m.* and *v.l.m.*). This size difference between the circular and longitudinal muscles accords with the conditions found in the adult (Brunotte,



TEXT-FIG. 28.

Slightly oblique transverse section of the first setiger of a young worm shortly after metamorphosis. Del. haem. This should be compared with the section shown in Text-fig. 6, and the general change of shape, &c., at metamorphosis noted.

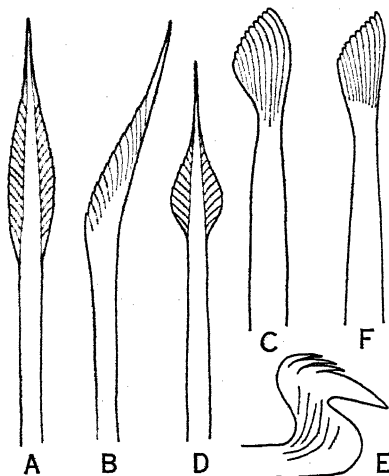
1888). Circular muscles also appear in the walls of the gut; they are fine, fairly widely spaced fibres.

After the final disappearance of the yolk the gut cells become highly vacuolated (Text-fig. 26). The internal surface of the gut is ciliated.

#### THE CHAETAE.

The first chaetae to appear are the dorsal bristles on the first and second setigers. Each bundle contains two bristles, one longer than the other, ending in a very fine point and winged, the margins of the wing being very finely toothed (Text-fig.

29 A and B). The other bristle is of peculiar shape (Text-fig. 29 C), having a somewhat clavate head, finely toothed and ridged. This is a purely larval bristle that appears only in the first two setigers. It persists for a time after metamorphosis, but is subsequently lost and apparently is not repeated. All bristles that later develop, before and after metamorphosis and



TEXT-FIG. 29.

A-E. Chaetae of *Branchiomma vesiculosum*.  $\times 1,430$ . A and B, two views of larval winged bristle; C, larval clavate bristle; D, type of winged bristle appearing after metamorphosis; E, larval uncinus of second setiger. F, larval clavate bristle of *Sabella pavonina*.

in both thoracic and abdominal segments, are winged types. They may be long with narrow wings, similar to the larval ones drawn in Text-fig. 29 A and B, but often are longer and slenderer, with lash-like tips, or they may be shorter, with small broad wings and long whip-like tips, as in Text-fig. 29 D. These latter ones are confined to the thoracic segments, at least up to the last stage to which young worms have been reared. They also do not appear until after metamorphosis, the first bristles in the third setiger and the third bristle in the first setiger, that is

sometimes developed in the last free-swimming stage, being of the narrow winged variety.

Uncini of the second and third setigers (the first never has an uncinus) appear later than the bristles, although in the third setiger the time lag is not as great as in the second. In the fourth and succeeding thoracic setigers uncini are, however, visible a short time before the bristles. The same seems to be true of abdominal segments. The uncinus of the second setiger is drawn in Text-fig. 29 E. The single large tooth is surmounted by three rows of smaller ones. Uncini of the succeeding thoracic and abdominal segments are similar, except that there are one or two more rows of these smaller teeth in the crest.

#### TUBE-BUILDING HABITS.

It has already been mentioned that when the larvae settle down to metamorphose they secrete around themselves a transparent thin-walled tube of mucus, the walls of which are later thickened by further additions of mucus. If metamorphosing larvae be removed from their clean tubes and put into a dish of fine sand, they will in a few hours form short sandy tubes. The sand grains are very loosely knit together, with large irregular gaps between them through which the worm can be seen, and it would seem that they are fortuitously collected by the sticky outside of the mucus tube which the larva secretes. At any rate, in spite of frequent examination during the time when the tubes were being formed, no larva was seen to select and handle sand grains in any way.

Whatever doubt there may be as to the manner in which the first sandy tube of the metamorphosing larva is made, it is quite certain that a few days after metamorphosis the worms increase the length of their tubes by means of definite building operations. Some young worms, less than two weeks after metamorphosis, had been living in a dish with a bottom sprinkled with sand and had tubes about seven millimetres long—considerably longer than they themselves—and 200–300 $\mu$  outside diameter. The tubes were firmly attached to the dish at one end, where there was sometimes a plain mucus tube that had apparently been secreted first. The sandy portions lay



horizontally on the bottom of the dish for most of their length, but in some a short piece at the unattached end turned vertically upwards. While moderately strong for their size, the tubes were easily broken up with needles. As before, the sand grains were very irregularly fixed to the outside of the transparent mucus tube or lining, that could be seen through the gaps between them. One of these tubes was selected and carefully cut with needles until it was only as long as the worm inside. This portion was placed in a cavity slide, sand grains added, and a cover glass applied. It was then possible to keep the worm under microscopic observation.

The animal, which had five to six setigers, immediately set about repairing the damage and in a few minutes had added several grains to its shortened tube. They were collected by the branchial pinnules, partly by the cilia on the latter causing the grains to travel along them towards the mouth and partly by bending movements of the pinnules. These are very pliable and can readily surround objects. No selection of grains was apparent, the nearest were seized indiscriminately, regardless of size. The mouth was applied to each grain as it arrived and it was then brought to bear, with very little adjustment, against the tube edge in any convenient position. Little or no attempt was made to make it fit in with those already there, excepting that if the edge had a big gap on one side the grain would very likely be put in there. Sometimes it would stick almost at once; presumably either the grain or the tube edge or both were previously coated with cement, but this cement could not be seen. Other grains would be held in position for a few seconds, steadied by the lips alone if small, by the basal parts of the pinnules if large. While holding it the worm passed the ventral glandular region of its body just behind the mouth backwards and forwards over the place of contact, the transverse row of cilia on either side of the region beating fast and vigorously during the operation, as if to spread the cement. After a few seconds of this treatment the hold on the grain was released and it remained in position, not immovably fixed but flexible at the joint. Another grain would then immediately be treated in the same way. None was discarded, except such as accidentally

fell from the pinnules. The worm would rest after fixing every half-dozen or so grains, the branchial cilia then being, apart from an occasional flicker, quite still. In an hour and a half, including rests, the worm built a portion of tube about half its own length.

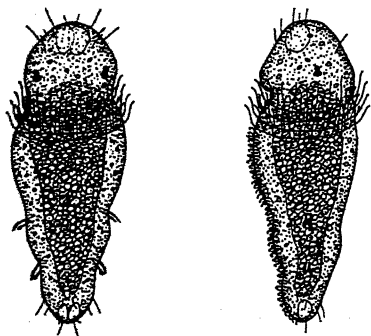
Some other specimens were observed to build in the same way. If removed entirely from their sandy tubes, they would, like metamorphosing larvae, soon renew them. In one instance a worm removed completely from its tube, constructed in under seven hours a loose rough tube about equal to its own length. This species evidently has no such difficulty in forming new homes at any time as have some worms, e.g. *Sabellaria* (Wilson, 1929). The latter are apparently never able to make new tubes for themselves should they be removed from their old ones, which had been originally begun by building on to the rims of larval tubes specially secreted as a foundation.

#### EARLY LARVAE OF *SABELLA PAVONINA* (SAVIGNY).

*Sabella pavonina* has in recent years been much used by experimental zoologists working on problems connected with regeneration (e.g. Berrill, Huxley, Gross), for which purpose it has proved to be specially suitable material. It would therefore seem that a detailed study of the development of *Sabella* should have been made in preference to the closely allied *Branchioma*. With this object in view I have spent much time and effort in attempting to obtain the larvae of *Sabella*, but without success. Although artificial fertilizations have been made in a variety of ways and at all times of the year, not a single egg has cleaved. Adults have been kept alive in the hope that they would shed their eggs naturally, but this has never happened. Fortunately, however, Dr. J. H. Orton succeeded in making a fertilization at West Mersea on May 31, 1927, brought the larvae to Plymouth a few days later, and presented them to me. By that time most were dead, but a few living larvae were picked out and a preliminary sketch made, reproduced in Text-fig. 30. The remaining larvae did not survive much longer and no later stages were obtained.

These larvae were rather smaller than those of *Branchioma*,

and the majority had four dark-red cup-shaped eye-spots, instead of only two, although some individuals had but two or three. It seems also that the head vesicle was double instead of single; it was so indicated in the sketch, which was made before *Branchiomma* larvae had been seen. Owing to the moribund condition of the *Sabella* larvae when they were preserved, cytological detail in the preparations is very indefinite and the only specimen that is at all reasonably clear supports, but does not absolutely confirm, the supposition that



TEXT-FIG. 30.

Larvae of *Sabella pavonina* about six days old. Preliminary sketches from life.  $\times 156$ . Dorsal view and view of left side. Actual length approx.  $250\mu$ .

the head vesicle in this species is double. If this should eventually prove to be correct, it will be an easy means of distinguishing these larvae from those of *Branchiomma*. As in the latter there are two anal vesicles. The prototroch appeared to have four rows or bands of cilia and there were a number of sensory cilia at either end of the body. A wide neurotroch was contained in a slight ventral trough. Two chaetigerous segments each had notopodial bristle bundles consisting of two chaetae. One of these was of the winged variety already familiar for *Branchiomma* (Text-fig. 29 A and B) and for all practical purposes indistinguishable from it. The other bristle (Text-fig. 29 F) was of the larval clavate type; it differed slightly in shape from that of *Branchiomma*. The whole larva was very yolky and

brownish yellow by transmitted light. According to Dr. Orton the colour had originally been pink, as are the eggs when viewed by reflected light. The few living larvae swam actively and rapidly forwards; they were contractile.

In their main features these *Sabellia* larvae were evidently very much like those of *Branchiomma*, and would probably have developed and metamorphosed in a closely similar manner. It seems justifiable to assume that the embryology of *Sabellia* will ultimately prove to be almost identical with that of *Branchiomma*.

#### DISCUSSION.

As this is the first occasion on which the development of a Sabellid has been described in any detail, a comparison of it with that of other Polychaetes is undoubtedly demanded. Unfortunately, when this is attempted the lack of precise information for many allied families becomes immediately apparent. There is a relatively large number of papers dealing with the larval forms of the closely related Serpulidae, particularly the early stages from the first cleavage to the trochosphere. Concerning the later stages there is little literature available, the most comprehensive account being that of Meyer (1888). The position is similar for most other families in which the larvae are known; the cleavage and trochosphere stages are often fully described, little or nothing being given about the later larvae and metamorphosis. Of the thirty-six families of Polychaetes (excluding the Archiannelida) forming the subject of Fauvel's monograph (1923 and 1927) we have detailed or moderately detailed information concerning the embryology of one or more species in only seventeen of them (Aphroditidae, Phyllodocidae, Hesionidae, Syllidae, Nereidae, Eunicidae, Ariciidae, Spionidae, Chaetopteridae, Cirratulidae, Capitellidae, Arenicolidae, Oweniidae, Sabellariidae, Terebellidae, Sabellidae, Serpulidae), but in no one family is there even moderately detailed knowledge of more than a very few species. In some instances it is only part of the development that is really well known. Of the remaining families we have a little, often imperfect and generally unsatisfactory, information

for ten of them (Alciopidae, Tomopteridae, Nephthydidae, Glyceridae, Magelonidae, Disomidae, Opheliidae, Maldanidae, Sternaspidae, Amphictenidae), and nothing whatever about the development of nine families (Chrysopetalidae, Pisionidae, Amphinomidae, Typhloscolecidae, Sphaerodoridae, Paraonidae, Chloraemidae, Scalibregmidae, Ampharetidae). With embryological knowledge for the Polychaetes as a whole in such an unsatisfactory state, it is scarcely justifiable to use such data as there are at present for the demonstration of relationships, which is one of the aims of embryological research. However, some interesting comparisons between the larvae of *Branchiomma* and those of neighbouring families can be pointed out, additional to those already considered in preceding sections.

The larva of *Branchiomma* is so well provided with yolk that it does not capture other organisms for food during pelagic life. This accumulation of yolk is probably also responsible for the absence of a large blastocoelic cavity. In one or both of these features, and also in general shape, it resembles the larvae of Capitellids, Cirratulids, and Terebellids perhaps more closely than it does those of Serpulids, although it differs from them and resembles Serpulids in the absence of a telotrochal ring. It resembles Serpulids too in the possession of the curious anal vesicles. According to Shearer (1911) *Hydroides* has one such vesicle, which later becomes partially constricted into two. Meyer (1888) shows two separate vesicles for *Psygmobranchus*. Shearer in his figures also shows vesicles in the apical region of *Hydroides* (fig. 2, Pl. 21) being particularly suggestive of the condition of the larval Sabellid head. Mead (1897) found five large vesicular bodies (his 'problematic bodies') in the head of *Amphitrite* larvae, which are probably homologous structures. He suggested that they were possibly homologous with the frontal bodies of *Nereis*.

When, however, we compare more fundamental structures it becomes quite clear that Sabellid larvae are more closely related to Serpulid larvae than to those of other families. In particular the rise of the branchial processes and the form of the metamorphosis (according to Meyer) are closely similar, with the

exception that in the Serpulid the larval tissue disappears by internal absorption instead of being thrown off externally. The development of the collar is evidently comparable, except that it is a much larger structure in Serpulid larvae than in Sabellid.

There is one very important difference between the course of Sabellid and Serpulid developments. In the latter, three thoracic setigers are formed practically simultaneously. The fourth and succeeding setigers as they appear are of the abdominal type, with dorsal uncini and ventral bristles. After several abdominal setigers have been formed the anterior ones are transformed into thoracic segments with dorsal bristles and ventral uncini until the worm possesses the full number of thoracic setigers characteristic of the adult (Malaquin, Faulkner). In the Sabellid, on the other hand, there is no very definitely marked stage with three setigers, and all adult thoracic segments are formed before any abdominal ones appear. This is all the more interesting because it has been shown that if an adult Sabellid is cut through in the abdominal region then, during subsequent anterior regeneration of the posterior piece, a varying number of abdominal segments are transformed into the thoracic type. 'The original setae and uncinigerous hooks in each segment drop out as their bases and muscles atrophy, while at the same time new setae and uncini appear, though dorsally and ventrally instead of ventrally and dorsally' (Berrill, 1931, p. 499). Berrill explains this by assuming that the segments regenerated anteriorly act as an 'organizer', bringing about the transformation of the abdominal segments situated posteriorly to them. However, later work by Huxley and Gross (1934) has shown that experimental cuts and wounds can affect reorganization in a great variety of ways, and they come to the conclusion that 'die Reorganisation nicht abhängig ist von einer "Organisatorwirkung" des Kopfes oder irgendeinem anderen differenzierten Körperteil'. In the present uncertainty of the significance of the manner in which regeneration and reorganization take place in the adult tissue, one hesitates to make theories as to the origin of the difference between the development of Serpulids and Sabellids.

The Sabellid larva is not highly specialized for pelagic life. The broad prototroch apart, it does not possess structures, such as long provisional bristles or a large spacious blastocoel, that are generally associated with a long life in the plankton. Its pelagic existence is for a Polychaete relatively short, or at least unprolonged, a feature it shares with a number of other similarly constructed and yolky larvae.

#### SUMMARY.

1. Larvae of *Branchiomma vesiculosum* Montagu were obtained from artificial fertilizations, and reared through metamorphosis and for some weeks afterwards.

2. The larvae are extremely yolky and do not feed until after metamorphosis. They swim by means of a broad prototroch, and are provided with a pair of cup-shaped eye-spots, a large head vesicle, and two anal vesicles. There is a broad neurotroch but no telotroch. The mouth is open but the anus is closed. In the last swimming stages rudiments of the adult branchial apparatus appear as a pair of lobed swellings, behind the eyes but in front of the prototroch. Behind the latter the collar rudiments appear. There are usually three or four chaetigerous segments marked out when metamorphosis sets in.

3. The metamorphosing larva settles on the bottom, and secretes for itself a tube of mucus. The prototroch and larval tissues (episphere) of the head clump together to form a large snout-like structure. This gradually breaks up into small pieces which are thrown off one by one as they are formed. With the loss of the prototroch and other larval head tissues the adult part of the head becomes joined on to the trunk. At the same time the branchial rudiments branch to form pinnules, which are directed forwards so that their bases overlap the eyes. The anus opens, and the young worm begins to feed. Metamorphosis occupies about four days.

4. The larval stages and the metamorphosis have been studied in histological, and to a limited extent in cytological, detail. Apart from the curious metamorphosis, which to some extent recalls that of *Owenia* and *Polygordius*, the development shows no striking features.

5. After metamorphosis the worm elongates by the addition of setigers in front of the pygidium. These are at first of the thoracic type (dorsal bristles, ventral uncini), but when the ninth and succeeding setigers appear they show abdominal constitution (dorsal uncini, ventral bristles) from the first. Thus no setigers change over from abdominal to thoracic constitution as they do in the development of young Serpulid worms.

6. Coincident with the loss of the neurotroch at metamorphosis the mid-dorsal line of the head and trunk of the young worm becomes ciliated to form the faecal groove of the adult. This strip of cilia continues on the ventral surfaces of the ninth and succeeding setigers, after passing round the right side between the eighth and ninth bristle segments. For a time the intersegmental groove between these segments is ciliated on the left side as well.

7. The branchial rudiments, which began to branch before metamorphosis, continue steadily to branch and grow afterwards. The pinnules are supported by an internal skeleton of thick-walled cells to the base of which the main dorsal and ventral longitudinal muscles of the body become attached. The most dorsal branch of each branchial rudiment, lying close to the mid-dorsal line, forms one of the so-called palps of the adult and is not supported by an internal skeleton.

8. The manner in which the young worms build their first sandy tube is described.

9. Early larvae of *Sabella pavonina* (Savigny) are described. They closely resemble those of *Branchiomma*.

10. The present position of embryological knowledge concerning the Polychaetes is very briefly summarized. It is shown that Sabellid larvae are more closely related to Serpulids than to those of any other family.

It is a pleasure to thank Dr. E. J. Allen for helpful encouragement. Dr. M. V. Lebour and Mr. A. J. Smith have occasionally looked after my finger-bowls while I have been away, and Mr. J. E. Smith once kindly made a successful fertilization for me. The late Professor J. H. Ashworth and Mr. C. C. A. Monro have helped by loaning or copying for me certain papers I should



otherwise have had great difficulty in procuring, and to them I am especially grateful. My best thanks are also due to my wife for typing the manuscript.

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## ADDENDUM.

Since this paper was sent to press an important work, "Regeneration and Reorganization in *Sabella*", by Gross and Huxley has appeared ('Arch. Entwicklungsmech.', Bd. 133, 1935). The authors strongly criticize the organizer hypothesis and suggest that the reorganization of parapodia from abdominal to thoracic type may be the result of a migration of tissue towards and into the regenerate, this migration initiating a process of alternative differentiation. A point of importance is that they clearly recognize that in anterior regeneration of *Sabella* there is generally produced the head, collar, and two setigers and no more. They regard this region of the adult as constituting a prothorax. This is, in fact, the region which forms the greater part of the larva of *Branchiomma* (and apparently also of *Sabella*), and whose setigers are specially characterized by the possession of larval clavate bristles, and perhaps also by the marked delay in appearance of uncini below the second bristle bundles. This delay is in contrast to the fourth and all succeeding segments, thoracic and abdominal, where the uncini are at least visible a little time before the bristles. The third setiger seems to be intermediate in the change over between these time relations (see p. 593).

In a still more recent paper on the same subject ("Reorganization and Regeneration in *Sabella*, I", 'Journ. Exp. Zool.', vol. 73, 1936), Berrill and Mees now conclude 'that a thoracic state is brought into being, not by the mere stimulus of cutting, but by the initial changes leading to the regeneration of new tissue that cutting does bring about'. A thoracic morphogenetic field is established and 'becomes extended as the blastema differentiates into a head'. It is suggested that a thoracic field is antagonistic to an abdominal field, and on the basis of this antagonism various regenerative phenomena can be explained.



# Planctosphaera and Tornaria.

By

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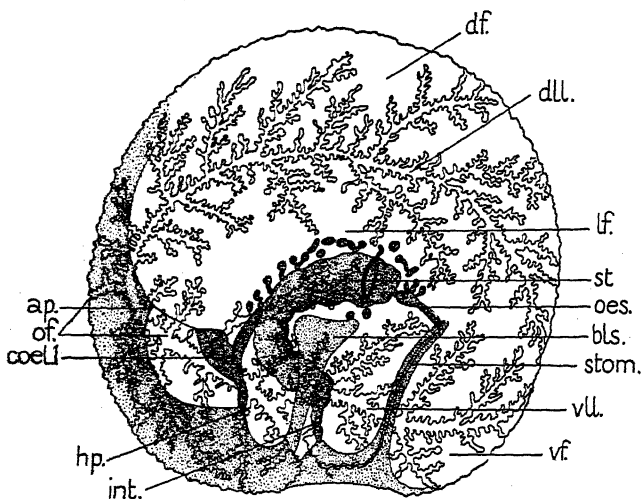
With 3 Text-figures.

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THE Norwegian 'Michael Sars' North Atlantic Expedition, 1910, collected in the Bay of Biscay two transparent, spherical animals, one of which was badly damaged. Mortensen recognized these animals as nearly related to the *Tornaria*, the larval form of the *Enteropneusta*, and they were therefore sent to Spengel, the well-known authority on *Enteropneusta*, for further examination. Spengel wrote a report on these animals, naming them *Planctosphaera pelagica*. This report, however, was published only in 1932, long after his death (1).

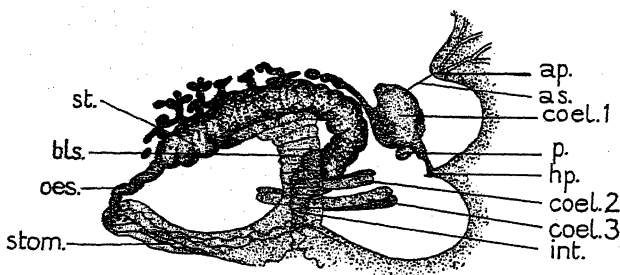
In his report Spengel described this animal as accurately as the material allowed and he gave some excellent drawings, illustrating the animal from all possible sides, as well as its internal organs. It appears that the larva, notwithstanding its spherical form, is bilaterally symmetrical. All its internal organs are situated in one quadrant, mouth and anus lie close together, and this side of the animal's body can best be designated as its ventral side. A narrow deep median groove extends over the ventral side, ending slightly beyond the posterior pole (Text-fig. 1).

The internal organs are similar to and even show a great similarity in form to those of *Tornaria* (Text-fig. 2). The alimentary canal consists of oesophagus, stomach, and intestine; the mouth is situated at the end of a long stomodaeal canal such as is also found in the large tentaculated *Tornaria*. The unpaired anterior coelom or hydrocoel with its hydropore opening to the exterior and connected by an apical string to the apical plate, the paired collar and trunk coelomic cavities,



TEXT-FIG. 1.

*Planctosphaera pelagica*. Lateral view. After Spengel.  
*ap.*, apical plate; *bls.*, blind-sac; *coel. 1*, anterior coelomic cavity;  
*df.*, dorsal field; *dll.*, dorso-lateral lobe; *hp.*, hydropore; *int.*,  
 intestine; *lf.*, lateral field; *oes.*, oesophagus; *of.*, oral field; *st.*,  
 stomach; *stom.*, stomodaeal canal; *vf.*, ventral field; *vll.*, ventro-  
 lateral lobe.



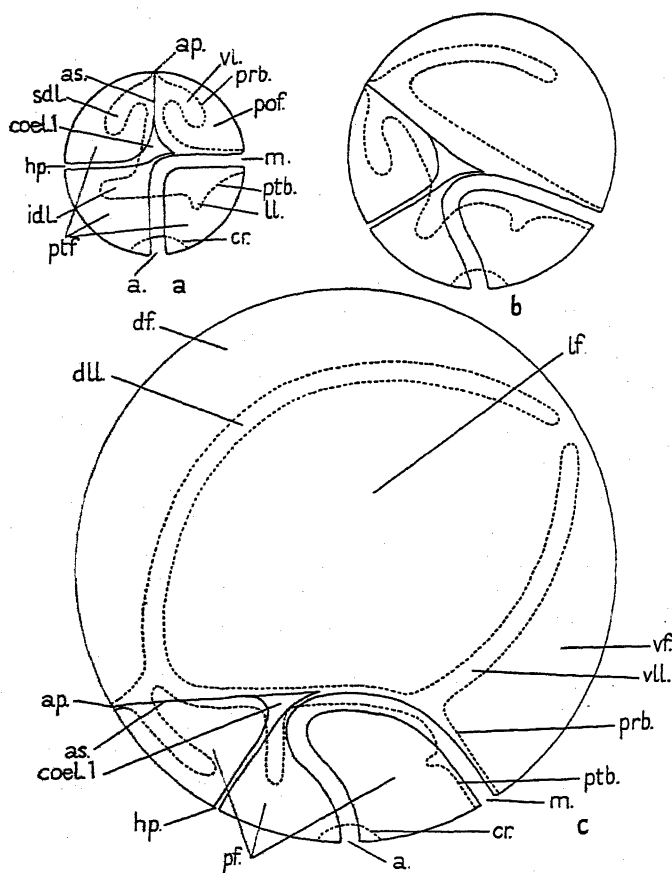
TEXT-FIG. 2.

*Planctosphaera pelagica*. Internal organs. After Spengel.  
*ap.*, apical plate; *as.*, apical string; *bls.*, blind-sac; *coel. 1*, anterior  
 coelomic cavity; *coel. 2*, collar coelom; *coel. 3*, trunk coelom;  
*hp.*, hydropore; *int.*, intestine; *oes.*, oesophagus; *p.*, pericardium;  
*st.*, stomach; *stom.*, stomodaeal canal.

lying in the form of narrow tubes at both sides of the intestine, and the pericardium are all similar to those of *Tornaria*. Externally there are also pre- and post-oral ciliated bands, as well as an apical plate; the latter, however, lacks the eye-spots of the *Tornaria*.

There is, nevertheless, a striking difference between *Planctosphaera* and *Tornaria*. In a median section of the *Tornaria* the pre-oral field, surrounded by the pre-oral ciliary band, occupies about one-quarter of the circumference. The other three-quarters is formed by the post-oral field, at least if the anal field, which only later during development becomes separated from the post-oral field by the ciliary ring, is regarded as part of the post-oral field. In *Planctosphaera* these relations are just the reverse. As *Planctosphaera* is much larger than most *Tornaria*, being about 10 mm. in diameter, one can imagine that the pre-oral field has greatly extended, while the rest of the body has retained the size it had in the *Tornaria*. I have tried to illustrate this relation between *Planctosphaera* and *Tornaria* in the diagrams of fig. 3.

The ciliary bands of *Planctosphaera* are much more intricate than those of *Tornaria*, and at first sight a comparison between the two animals seems hardly possible. In *Tornaria* the ciliary bands form primary and secondary lobes; in *Planctosphaera* tertiary and even lobes of higher orders are added to these. In the *Tornaria* the oral field, extending itself between the two bands, has about the same dimensions as the pre- and post-oral fields. Only in the large tentaculated *Tornaria* is the oral field much narrower, so that the pre- and post-oral ciliary bands are parallel to each other, and in the lobes the ascending and descending loops of one band are also parallel. In *Planctosphaera* this is even more marked; the oral field is reduced to a narrow strip with a ciliary band on each side. The lobes of the oral field, formed by the loops of the ciliary band branch over the whole surface of the animal, dividing the pre- and post-oral fields into different parts. It is quite easy to recognize the main part of the oral field, delimited on the one side by the pre-, on the other by the post-oral ciliary band. Starting at the mouth, in the depth of



TEXT-FIG. 3.

Diagram illustrating the relation between *Tornaria* and *Planctosphaera*. (a) Spherical *Tornaria*; (b) hypothetical intermediate form; (c) simplified *Planctosphaera*. *a.*, anus; *ap.*, apical plate; *as.*, apical string; *coel. 1*, anterior cloeomic cavity; *cr.*, ciliary ring; *df.*, dorsal field; *dll.*, dorso-lateral lobe; *hp.*, hydropore; *idl.*, inferior dorsal lobe; *lf.*, lateral field; *ll.*, lateral lobe; *m.*, mouth; *pf.*, posterior field; *pof.*, pre-oral field; *prb.*, pre-oral ciliary band; *ptb.*, post-oral ciliary band; *ptf.*, post-oral field; *sdl.*, superior dorsal lobe; *vf.*, ventral field; *vl.*, ventral lobe; *vll.*, ventro-lateral lobe.

the stomodaeal canal, we see that the oral field, which in this region is very narrow, turns at first in a lateral direction and then proceeds in a curve in the direction of the posterior end of the body. Not far from the posterior end it turns again at right angles, and then the oral field of the one side of the body meets its fellow of the other side at the posterior pole. This place thus corresponds to the apical plate of the *Tornaria*.

Near the posterior side of the body, where the oral field turns at right angles towards the posterior pole, the main part of the oral field seems to be continued into a large branch or lobe, formed by the pre-oral ciliary band only. This lobe runs along the dorso-lateral side of the body and ends very near its anterior pole. A similar large lobe, running along the ventro-lateral side, branches off from the oral field not far from the entrance of the stomodaeal canal. The course of these branches and all the ramifications of the oral field have been adequately described by Spengel.

The lobes made by the pre- and post-oral ciliary bands of the *Tornaria* are arranged in such a way around the apical plate that the animal is nearly radially symmetrical in external appearance. A similar radial symmetry is exhibited by *Planctosphaera* in the arrangement of the two dorso-lateral and the two ventro-lateral lobes of the oral field, but in *Planctosphaera* the centre of symmetry is found at the anterior pole of the body, just opposite the apical plate. No doubt this radial arrangement of the ciliary bands is of great importance for the locomotion of the animal. Presumably it swims with the anterior pole of the body forwards.

By the ciliary bands the body is divided into several fields. The designations given by Spengel to these fields do not seem to me to be very adequate. Spengel calls the large field between the two dorso-lateral lobes 'Hinterfeld', but as it extends over the whole dorsal area of the body from the posterior to the anterior pole, dorsal field would be a better name for it. In the same way the field between the two ventro-lateral lobes is best called ventral field and not 'Vorderfeld', even though it does not extend over the whole ventral surface of the animal but only over the anterior half of it. Between the dorso- and



ventrolateral lobes are found the lateral fields or 'Seitenfelder' of Spengel. Finally there is the area between the mouth and the apical plate, surrounded by the post-oral ciliary band. Spengel calls this area 'untere Hinterfelder'. These two 'Felder' are separated from each other by the median groove, not by a ciliary band, so that in reality they form only one field, which is best described as the posterior field. This posterior field corresponds to the whole post-oral field, including the anal field, of the *Tornaria*, whereas the dorsal, ventral, and two lateral fields, which merge into each other at the anterior pole, correspond to the small pre-oral field of the *Tornaria*.

It is possible that *Planctosphaera* has also a ciliary ring separating the anal from the post-oral field. At least Spengel was able to discern a dark ring surrounding the anus. This ring, however, is incomplete; it is open at the side of the mouth, and no cilia could be detected on it.

Text-fig. 3 also shows very clearly in what way the lobes of *Planctosphaera* may be related to those of *Tornaria*. The large dorso-lateral lobes correspond without doubt to the ventral lobes of *Tornaria*, whereas the ventro-lateral lobes of *Planctosphaera* are not represented in *Tornaria*. The post-oral ciliary band of *Tornaria* forms three primary lobes, viz. the superior and inferior dorsal lobes and the lateral lobe. The determination of these lobes in *Planctosphaera* cannot be done with the same degree of certainty. However, in this animal two pairs of large lobes penetrate into the posterior field, one near the apical plate and running parallel to the median groove and the other from the sides. If they correspond to any of the tornarian lobes, it is probable that they represent the superior and inferior dorsal lobes. A small lobe near the entrance to the stomodaeal canal might be the lateral lobe of *Tornaria*.

Of the internal organs the position of the unpaired anterior coelomic cavity is peculiar. In *Tornaria* this cavity is found at the anterior end of the stomach near its junction with the oesophagus; in *Planctosphaera* it is situated near the posterior end of the stomach. According to Spengel this position can be explained in two ways. It may be supposed that the

coelomic cavity was separated from the top end of the archenteron as in *Tornaria* and has shifted from there along the dorsal side of the stomach to its more posterior position. In this case, this shifting should have taken place directly after the separation and before the hydropore and the apical string, connecting this cavity with the epidermis, are formed. Another possibility, and one which is more favoured by Spengel, is that the whole wall of the archenteron might form the coelomic vesicle, and that under the influence of the apical plate the anterior part is activated to do so in the *Tornaria* and the more posterior part in *Planctosphaera*. To me the following explanation seems much more likely. The coelomic cavity is separated off from the top end of the still small archenteron in the same way as in the *Tornaria*. After this separation, and as a direct result of the enormous extension of the pre-oral side of the body, the archenteron grows out to form the long stomach of *Planctosphaera* and to connect with the stomodaeal canal for the formation of the mouth. The position of the anterior coelom in relation to the other coelomic cavities is the same in both larvae.

The occurrence of one organ in *Planctosphaera* is remarkable, were it only for the fact that it is not found in *Tornaria*. From the ventral side a pair of blind-sacs penetrate into the body from the surface. They converge towards the interior, their external openings being farther separated from each other than their tops. These tops lie at the sides of the stomach though not actually touching its wall, and at their tops the sacs are widened out in an anterior direction.

Though in several respects *Planctosphaera* is very similar to the *Tornaria*, yet it differs too much from the *Tornaria* in the general form of its body and also in the presence of these blind-sacs to be a larval *Enteropneust*. Moreover, by the work of Stiasny we know rather accurately to which genera of adult *Enteropneusta* the different types of *Tornaria* belong. With only one exception the tornarian type is known of all genera undergoing an indirect development. As all genera of *Enteropneusta* as well as all types of *Tornaria* exhibit similar characters, there seems no

justification for regarding *Planctosphaera* as the hitherto unknown larva of *Schizocardium*.

The difference between *Planctosphaera* and the larva of *Cephalodiscus* is even greater, and this makes it very unlikely that *Planctosphaera* is the unknown larva of *Rhabdopleura*. *Planctosphaera* is also comparatively much larger than *Rhabdopleura*, so that for this reason alone it can hardly be its larva.

Spengel, therefore, is quite right when he puts *Planctosphaera* in an order by itself. The class of the Hemichordata should then be divided into three orders, viz. Enteropneusta, Pterobranchia, and a third, which could provisionally be called *Planctosphaeroidea*, until the adult animal is found.

Little can be said about the form of this adult animal. It would also be impossible to visualize an adult Enteropneust, if only a *Tornaria* was known. In its general form I suppose it will bear more resemblance to an Enteropneust than to any other animal. Most likely the great extension of the pre-oral part of the body is only a larval character. As in Enteropneusta, the ciliary band will disappear during metamorphosis and the body of the adult animal will be formed from the ventral part of the larval body, where the different organs are situated.

The two blind-sacs penetrating into the larval body seem to be of great importance. Apparently they are not larval organs and have no functions at all during larval life. These sacs can only be the rudiments of some organ of the adult animal. It is only the question what organ that can be. Spengel tried to solve this problem, and in accordance with his firm conviction that the Enteropneusta are not related at all to the Chordata, he compared this organ with the ectodermal invagination of *Actinotrocha*, which becomes everted to form the body of *Phoronis*. This invagination, however, is from the beginning a median organ, attached by the mesentery to the intestine. The blind-sacs of *Planctosphaera*, on the other hand, are paired; they are not attached to the intestine by a mesentery, which is only formed later. If they were

capable of eversion as in *Phoronis*, they would have to fuse with each other first and a mesentery would have to be formed between these fused sacs and the intestine. Spengel accepted this and imagined that by the eversion and further outgrowth of the sac the anus would come to be on the other side of the mouth, in which case the animal would acquire a form more or less similar to that of *Cephalodiscus*.

Most students of *Enteropneusta*, following Bateson, accept the chordate affinities of this group of animals, and I think in this direction lies the solution of these remarkable organs. The only other structures with which they show a great similarity and with which I can identify them are the rudiments of the peribranchial cavity of *Ascidians*. This cavity also arises as two separate blind-sacs penetrating from the outside into the body and lying with their blind ends at the side of the intestine. Only later their openings fuse to form the egestion aperture of the adult animal. I can only come to the conclusion that these blind-sacs are the homologues of the peribranchial cavity of *Tunicates* and therefore of the atrium of *Amphioxus*. Thus *Planctosphaera* would be the larva of an animal, either sessile or free living but more probably the latter, nearly allied to the *Enteropneusta* and exhibiting most of the characters of *Enteropneusta*, but by the presence of an atrial cavity intermediate between this group of animals, the *Tunicates* and *Amphioxus*. I wonder when this animal will be found, and what it will look like!

#### LITERATURE

1. J. W. Spengel, *Planctosphaera pelagica*. Report on the scientific results of the 'Michael Sars' North Atlantic Deep-Sea Exped., 1910, vol. 5. Bergen, 1932.



# Observations on the Effect of the Ultra-centrifuge on some Free-living Flagellates.

By

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With Plates 30 to 32 and 1 Text-figure.

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## I. INTRODUCTION.

THE cytoplasmic components and inclusions of free-living flagellates have been the subject of many investigations. It is, however, a subject which has given rise to much diversity of opinion, and it is often difficult to reconcile the observations of different workers, sometimes even to compare their results.

The development of air-driven ultra-centrifuge by J. W. Beams and his associates (1930 and 1933) has proved a valuable means of approach to the investigation of the cytoplasmic components of various types of cells of both animals and plants. Owing to the difference in specific gravity, the proto-

plasmic inclusions and components may frequently be separated from one another and stratified in various parts of the cell. It is also a fruitful method of distinguishing between true pre-formed inclusions and those which may be artifacts produced by fixation.

That the centrifuge will prove to be of great value in the study of the Protozoa and Protophyta is certain, though up to the present little use has been made of this technique. McClendon (1909), Harvey (1931), and King and Beams (1934) have noted that in strongly centrifuged *Paramecium*, crystals are readily thrown down to the centrifugal pole, as is also the nucleus in many cases. Harvey (1931) found that in *Stentor* the chloroplasts also pass to the heavy pole.

Having abundant cultures of *Euglena* and some other organisms we thought it might be interesting to observe the effect of the ultra-centrifuge upon them. *Euglena* is a genus whose cytology has been much studied. As regards the general form, the nucleus, chloroplasts, paramylum grains, stigma, and flagellar structure there is fairly general agreement. The controversial points are the 'vacuome' (neutral red bodies), volutin granules, Golgi bodies, and mitochondria. It is the purpose of this paper to study the stratification of the components and inclusions in centrifuged organisms, to investigate their relative densities and to see if this stratification can throw light on any of the debatable problems of their cytology. To anticipate the results we may state that the heaviest materials, which collect at the centrifugal pole, are the paramylum grains and neutral red stainable bodies; then the chloroplasts, while the other pole of the organism is filled with cytoplasm containing many small granules which are probably the mitochondria. In addition we have evidence to support the view that the contents of the 'vacuome' are identical with the volutin granules of Baker (1933). Preliminary observations on the effect of the ultra-centrifuge on *Menoidium* and *Chilomonas* have also been recorded.

We wish to thank Professor J. Brontë Gatenby for his valuable advice and many helpful criticisms, and Mr. R. Brown for constructing the apparatus which supplied the air-pressure.

## II. MATERIAL AND METHODS.

The organism which we have studied most fully is a species of *Euglena* obtained in hay infusion cultures.

*Chilomonas paramecium* Ehrb. and *Menoidium* sp. have also been employed.<sup>1</sup>

In all cases the organisms have first been studied in the living condition, with and without the use of vital dyes, before being centrifuged. The *Euglenae* do not stain readily with vital dyes, certainly with neutral red which was most used. Hall's alcoholic neutral red method of staining (1929 *b*, 1931) was attempted, but was not very effective. We find it necessary to allow the organisms to stand in a fairly strong aqueous solution (approximately 1/5,000 neutral red in water) for some hours to obtain staining of the 'vacuome'. *Menoidium* was treated in the same way and is even slower in taking the stain. *Chilomonas*, however, stains rapidly (5-15 minutes) by either Hall's alcoholic method, or when a watery solution of neutral red is added to the culture. (In the latter case 1/10,000 or less is effective.) As far as has been observed the cultures showed no ill effects when the aqueous neutral-red solutions were added to them.

Having satisfied ourselves as to the normal morphology, the organisms were placed in a gum arabic solution of approximately the same density, or left in their own culture medium, and were centrifuged in the air-driven ultra-centrifuge, recently described by Beams, Weed, and Pickles (1933), for 2½-3 minutes at about 100,000 times gravity. This was found to give excellent results for *Euglena*, and was also used for *Menoidium*, but was much too powerful for *Chilomonas*, which disintegrated at this pressure. The latter was therefore centrifuged for the same time at from 40,000 to 50,000 times gravity.

<sup>1</sup> Unfortunately we are unable to identify the species of *Euglena* with certainty. It appears to be a form close to *Euglena gracilis* Ehrb. We give a description of the organism below (p. 618). The species of *Menoidium* is also uncertain; for it is somewhat too long and slender to be typical of the common species *Menoidium incurvum*, but it is almost certainly a variant of this form. We would like to thank Mr. C. Dobell, F.R.S., for examining our cultures and giving us the benefit of his advice.



For centrifuging and fixation large quantities of the organisms were collected with the aid of a hand centrifuge. In all cases, as well as fixing material exposed to the ultra-centrifuge, controls were made of organisms not subjected to this force.

The fixatives employed were sublimate acetic; Bouin's, Schaudinn's, Champy's, and Regaud's fluids, all subsequently stained by iron alum haematoxylin. Some material fixed in Schaudinn's fluid was also stained by Feulgen's method.

Meyer's methylene-blue test for volutin was used as described by Baker (1933). The organisms were fixed in Schaudinn's fluid and after washing were placed in a 10 per cent. solution of methylene-blue for ten minutes, after rinsing were treated with a 1 per cent. solution of sulphuric acid for half a minute, upgraded, and mounted in the usual way.

Da Fano's and Cajal's silver methods and also Kolatchev and Mann Kopsch techniques were used. In the latter cases osmication was carried out for 7-12 days at 35° C., and in some cases was afterwards bleached by treating with potassium permanganate and oxalic acid. The osmic vapour method as described by Hall (1931) was also followed. Other vital stains and fixation techniques will be described where appropriate in the text.

### III. OBSERVATIONS ON *EUGLENA* SP.

#### (i) General Morphology of Normal and Centrifuged Organisms.

Before the effect of the ultra-centrifuge on *Euglena* can be interpreted profitably, it will be well to discuss briefly the appearance of the control organism.

The flagellated, motile stages of *Euglena* (fig. 1, Pl. 30) are spindle-shaped, with greatest length about 63 $\mu$ , and maximum width about 10 $\mu$ . A single flagellum emerges from a short gullet at the anterior end of the body. The gullet opens into a reservoir and the flagellum bifurcates at the anterior end of the reservoir, each part terminating on its posterior wall. In the cytoplasm just anterior to the reservoir is a red to yellow coloured stigma composed of numerous, well-defined pigment

granules. In a few cases, particularly non-motile forms, bodies resembling the pigment granules of the stigma have been seen scattered in the cytoplasm.

There is a single nucleus, with nucleolus, generally located near or just posterior to the centre of the organism. Chlorophyll is present in the form of 12-25 elongated and somewhat flattened chloroplasts. The organisms have a brilliant green colour, but owing to the quantity and size of the paramylum grains it is difficult to see the outlines of the chloroplasts, or to count the number with certainty. Paramylum is in the form of oval, highly refractile bodies. Both paramylum bodies and chloroplasts are more or less evenly distributed throughout the cytoplasm with the exception of the extreme anterior end, which alone possesses fewer of these bodies and appears clearer.

When *Euglenae* are supravitaly stained with neutral red many globules make their appearance in the cytoplasm (fig. 1, Pl. 30). Smaller refractile bodies which we have been unable to stain clearly with vital dyes, and which, like the neutral-red bodies, are evenly distributed in the organism, we have tentatively interpreted as mitochondria.

After centrifuging, a marked stratification of the parts takes place, as can be seen in figs. 2-6, Pl. 30. When examined rapidly after centrifuging, most of the organisms have contracted into a more or less oval shape and are non-motile. Those that are in the fully elongated condition throughout generally do not show such clear-cut stratification. However, restoration of motility soon occurs, followed later by redistribution of the components, so that after a time (6 hours—overnight) one can no longer perceive that the organisms have been exposed to the centrifugal force. It would be interesting to follow this process of redistribution in greater detail.

The stratification of the parts within the *Euglenae* takes place irrespective of the morphological polarity of the organism. In other words, it is a matter of chance whether the heavy materials are displaced to the posterior (fig. 2, Pl. 30) or anterior end (fig. 3, Pl. 30), or even laterally (fig. 6, Pl. 30). Presumably it depends on the orientation of the organisms to the centrifugal force at the time when centrifuging begins.

## (ii) Paramylum.

The appearance of living centrifuged *Euglenae* is shown in figs. 2-6, Pl. 30. It can be seen that the paramylum bodies (p.) have been moved and are piled up together at one pole, the centrifugal pole, which, as already stated, may be the morphological anterior, posterior, or lateral part of the organism. Thus it may be seen that the paramylum bodies, like the starch grains in plant-cells (Beams and King, 1935), constitute the heaviest material. The paramylum bodies in centrifuged *Euglenae*, though closely packed, always remain discrete. In fixed preparations we have not succeeded in staining them, but they can nearly always be seen as clear areas (figs. 8-15, Pls. 30-31), and after sufficiently long impregnation by Mann Kopsch's method some of the bodies may be blackened (fig. 11, Pl. 31) though this may be removed by bleaching.

## (iii) 'Vacuome' and Volutin.

As already indicated a long immersion of *Euglenae* in neutral red (1:5,000 approx.) stains numerous globules a bright red colour (fig. 1 N.R., Pl. 30). Pierre Dangeard in 1924 was the first to show the presence of the so-called vacuome in *Euglena*, in the form of small globules which stain with neutral red. Similar bodies have been noted in *Euglena* (various species) by Grassé, 1925; Pierre Dangeard, 1928; Hall and Dunihue, 1930; Hall, 1931; and Baker, 1933.

Hall and his pupils have shown the presence of bodies which stain with neutral red in many protozoa (Hall in *Peranema*, 1929 a; *Chromulina*, *Chilomonas*, and *Astasia*, 1930; Hall and Dunihue in *Vorticella*, 1931; Hall and Loefer in *Euglypha*, 1930; Hall and Nigrelli in *Chlamydomonas*, 1931), and such bodies are regarded as homologous with the Golgi bodies of the metazoa, for the authors state that the neutral-red bodies blacken when the organism is exposed to osmic vapour, and also, in preparations preserved by classical Golgi apparatus methods, granules which are equivalent in size, number, and distribution are blackened.

Hall and Dunihue (1930) in addition consider that the 'vacuome' in *Chlamydomonas* and *Chlorella*, and Hall

and Nigrelli (1931) in *Chlamydomonas*, acts as a storage for starch and gives a blue reaction with iodine-potassium iodide. Baker (1933), however, does not agree that the neutral-red bodies of *Euglena* represent the Golgi bodies, but considers that the contents of the vacuome (when alkaline) are identical with the volutin granules shown after the specific methylene-blue staining, and similar to the volutin found in so many Protozoa.

Figs. 2-6, Pl. 30, show the distribution of the neutral-red bodies after centrifuging vitally stained organisms. It can be seen that these globules are confined to the same general area as is the paramylum. It is a conspicuous feature that the paramylum and neutral-red bodies always stratify in the same layer; though as fig. 3, Pl. 30, shows the neutral-red bodies may not pass quite so far to the centrifugal pole as do the paramylum grains. These results can only be interpreted to mean that the two components have a similar relative specific gravity.<sup>1</sup>

Generally the periphery of the neutral-red bodies stains rather more intensely red than does the central region. With long immersion in neutral red (overnight) the globules increase somewhat in size, so that a process of segregation may occur after the initial staining of the true pre-formed body. There appears to be a maximum limit to the amount of segregation, for the globules do not increase indefinitely in size.

When organisms, stained intravitaly with neutral red, are exposed to osmic vapour for a fairly long time (2-3 days), we find that generally, though not invariably, blackened rings, discs, and, less frequently, spherules are revealed (fig. 14, Pl. 31). In centrifuged material these rings are confined to the centrifugal pole (fig. 15, Pl. 31), i.e. the same area as the paramylum, and therefore the region which contained the neutral-red-stainable bodies in the living organism. It is obvious that the objects blackened by osmic vapour represent at least the periphery of the spherules that were shown previously by intravital staining with neutral red.

<sup>1</sup> It is interesting to note that Mr. R. Brown in some unpublished investigations carried on in this laboratory, has lately found in centrifuged ganglion cells of the rat that the neutral-red bodies are light and pass to the centripetal pole.

It is worth mentioning that we do not find the rapid blackening of the neutral-red bodies which Hall (1931) states to have occurred when he exposed *Euglena gracilis* to osmic vapour.

Organisms not previously stained with neutral red were similarly treated with osmic vapour, in order to determine the possible effect of the presence of the vital dye on the subsequent fixation with osmic vapour. In such unstained organisms we have not found the rings or discs referred to above, and can only conclude that neutral red induces their appearance (see also Douglas, Duthie, and Gatenby, 1933). However, the 'vacuome' or neutral-red-stainable bodies can sometimes be seen in such preparations as small spherules, rather like those shown in fig. 12, Pl. 31 (Mann Kopsch preparation).

In Kolatchev and Mann Kopsch preparations we have not always succeeded in showing the 'vacuome' whether these bodies were previously stained intravitaly or not. As in the case of the osmic vapour method, deeply blackened rings and crescents were only seen after preliminary treatment of the organisms with neutral red (fig. 13, Pl. 31). Fig. 12, Pl. 31, shows *Euglena* fixed by Mann Kopsch technique in which the 'vacuome' is in the form of light globules very similar to those depicted by Baker with the Kolatchev method in his figs. 36 and 38. Whether ring-like or spherical these bodies are confined to the centrifugal pole in centrifuged organisms. Regaud's method sometimes showed globules confined to the same region; while iron alum haematoxylin staining following fixation in Bouin's fluid or sublimate acetic may stain irregular masses, probably corresponding to the neutral-red-stainable bodies, in the interstices between the clear paramylum grains.

Baker has shown that if an alkaline reaction is given by the neutral-red bodies, organisms from the same culture, treated by Meyer's volutin method, give a metachromatic reaction of granules having the same size and distribution as the bodies stained red in living *Euglenae*.

We have found that in control material prepared by this method, bodies are scattered at random throughout the cytoplasm, whereas in centrifuged *Euglenae* they are clearly stratified (figs. 17 and 18 n.r., Pl. 32). There is no doubt that

these bodies are confined to the centrifugal pole, for one may sometimes see the chloroplast band (c.) with the nucleus lying to its distal side. As will be shown below, the nucleus lies towards the centripetal side of the chloroplast band.

The bodies give the metachromatic stain of a purple-to-reddish colour, sometimes with a bluish periphery. With toluidin blue, similar bodies are revealed. There is no doubt that the granules are of about the same size and are affected in the same way by the centrifuge as the neutral-red globules of living *Euglenae*. We therefore have evidence to confirm Baker's contention that the bodies which stain by this technique correspond to the contents of the globules which stained intravitaly with neutral red. We did not obtain any starch reaction of the 'vacuome' with iodine.

#### (iv) Peripheral Bodies.

*Euglenae* preserved by fixatives containing osmic acid show spherical bodies coloured a brownish or black colour, lying close to the surface of the organisms (figs. 7-15 x., Pls. 30-31). They could not be stained intravitaly.

In organisms exposed to osmic vapour these are the first structures to darken and it might easily be thought that they were the neutral-red bodies. That they are not is shown by examination of centrifuged material, for unlike the neutral-red globules these peripheral bodies are unaffected by the centrifuge. Their position and distribution is exactly the same before and after being exposed to this force (fig. 15, Pl. 31).

As mentioned above osmic vapour may also show, in the same organism, the neutral-red bodies as blackened rings, which are, however, confined to the centrifugal pole (fig. 15, Pl. 31). In Mann Kopsch preparations of centrifuged material a similar dispersal of both peripheral bodies and 'vacuome' may be seen (figs. 12-13, Pl. 31). The peripheral globules, unlike the bodies stainable with neutral red, are almost invariably darkened by any osmic acid method of fixation. They are always seen as homogeneously stained spheres.

These bodies do not appear to have been mentioned apart from other structures by previous workers. It may be that they

are confined to the culture of *Euglena* we have studied, or they may have been present in those organisms investigated by other workers, but confused with some of the other components.

Clearly they are not the 'brown bodies' mentioned by Baker (1933). Similar 'brown bodies' (degenerating chloroplasts) have been seen by us, but have not the same size, distribution, or regularity in appearance (fig. 7, Pl. 30).

Dangeard (1928, quoted from Dubosq and Grassé, 1933) states that there is a peripheral secretion of mucin in the form of globules in *Euglena gracilis*, which we thought might correspond with our peripheral bodies. The latter, however, could not be stained by mucicarmin following Zenker's fixation.

We are quite undecided as to the nature of these bodies. Formalin fixation followed by impregnation with osmic acid failed to show them, and organisms fixed in the same manner and stained by Scharlach R, Sudan III, or Nile-blue sulphate gave no definite evidence of staining of these bodies. We have never seen them in the material fixed in sublimate acetic, Bouin's or Schaudinn's fluid with any of the stains we have used; nor do they stain by iron alum haematoxylin following Regaud's method. We can only say that they are spherical bodies lying close to the periphery, readily darkened by osmic acid (though this can easily be removed by bleaching), and that they are not affected by the centrifuge.

#### (v) Chloroplasts.

After centrifuging, the chloroplasts (c.) are generally gathered into a belt separating the paramylum and neutral-red bodies on one side from the clearer cytoplasm containing the probable mitochondria on the other (figs. 2-6, 8-13, Pls. 30-31). In living *Euglenae*, stained with neutral red, stratification is beautifully clear; a brilliant green band lies in the middle with a clear area on one side and a reddish region on the other (owing to the massing of the neutral-red bodies).

In some cases the chloroplasts are so closely packed together that it is impossible to discern their outlines (figs. 2-4, Pl. 30), but there is no evidence that the chloroplasts ever burst;

redistribution can always take place. The chloroplasts do not always form such a conspicuous belt, but may be scattered rather irregularly along the centripetal side of the paramylum (fig. 5, Pl. 30).

After osmication the chloroplasts are darkened to various degrees, depending on the length of impregnation, but bleaching can remove this blackening. Harvey (1931) in *Stentor* mentioned that the chlorophyll moved to the heavy pole, Motier (1899), whose observations one of us (H. W. B.) has confirmed (unpublished), found the chloroplasts of certain plant-cells to be thrown down to the centrifugal pole. From our present experiments on *Euglena* it is obvious that the chloroplasts are denser than the surrounding cytoplasm, but not so dense as the paramylum and neutral-red-stainable bodies, hence they cannot be fully displaced to the centrifugal pole.

#### (vi) Nucleus.

In centrifuged organisms the nucleus is found to lie in much the same area as the chloroplasts, or on their centripetal side (figs. 13 and 17, Pls. 31-32). The nucleus is not shown in most of the drawings as the chloroplasts tend to veil it; it has, however, been carefully studied after staining by Feulgen's method, when the light green counterstain shows the chloroplasts without hiding the mauve-stained nucleus. In some Regaud preparations the nucleolus can be seen displaced to the centrifugal side of the nucleus.

#### (vii) Mitochondria.

In the clearer cytoplasm at the centripetal pole of centrifuged *Euglenae* small granules can generally be seen both in living organisms (figs. 2 and 3 m., Pl. 30) and after fixation by the Kolatchev, Mann Kopsch, and Regaud methods (figs. 8, 11, and 12, Pls. 30-31). They are easily seen after good Mann Kopsch fixation, subsequently slightly bleached, when they are greyish.

We have not succeeded in staining these granules clearly intravitaly with Janus green B, so hesitate to affirm that they are unquestionably mitochondria. They do, however, resemble



the bodies identified by Baker (1933) in *Euglena gracilis* as mitochondria and also those shown by Brown (1930) in the same species, as the 'chondriome'. Hall (1931), also working with this species, found rod-shaped as well as spherical mitochondria, both staining vitally with Janus green B; while Grassé (1925) in *Euglena proxima* states that he had no success with intravital staining with this dye, but that various fixatives reveal rod-shaped bodies up to  $5\mu$  long which are probably to be interpreted as mitochondria. The effects of the centrifuge on these probable mitochondria of *Euglena* can be compared directly with that obtained by Beams and King (1935) in plant cells, where the mitochondria were not so dense as the starch grains and accordingly were forced to take up a position centripetal to them. Likewise in *Euglena* the mitochondria seem to be of a lower specific gravity than either paramylum, neutral-red bodies, or chloroplasts.

#### (viii) Stigma, Reservoir, and Flagellar Apparatus.

The centrifuge appears to have no effect on these parts. Owing to the rounding up of the organism at the time of centrifuging they may be temporarily difficult to see, but in no case do they lose their orientation with anterior end of the organism, regardless of the direction of the centrifugal force (figs. 2, 3, 10, &c., Pls. 30-31).

There is some suggestion that the centrifuge may have an effect of separating the granules of the stigma (fig. 4, Pl. 30), but this is uncertain as in non-motile and sometimes even motile control forms the stigma may be found broken up.

#### (ix) Golgi Bodies.

This is a most controversial subject, and it is not our purpose to do much more than state the theories of other investigators as briefly as possible. The centrifuge method has not enabled us to throw much new light on the matter.

Grassé (1925 and 1926) and Dubosq and Grassé (1933) identify the stigma of *Euglenoids* as being homologous with the

parabasal body of parasitic flagellates and sponges, and so with the Golgi bodies of other protozoa and the metazoa. This interpretation of the stigma has not found much favour with other workers (see Mangenot, 1926, &c.).

We have found that the stigma blackens readily in osmicated material, but that this is quickly lost with bleaching. Sigot (1931) blackened a series of small globules in *Euglena gracilis* lying close to the periphery of the reservoir and stated that each globule was composed of a chromophilic cortex and a chromophobic medulla. No other workers seem to have observed such structures, but we find that in some Mann Kopsch preparations (figs. 11, 12, 16, Pls. 31-32) we have obtained a blackening of the outside of the reservoir and of numerous small globules which appear to be close to, or be part of this region. This blackening resists bleaching very effectively, and, as can be seen in fig. 16, Pl. 32, and Text-fig. 1, everything else in the organism can lose its colour while the reservoir remains intensely black.

We have already referred to Hall's interpretation of the Golgi apparatus nature of the 'vacuome'. We cannot agree with this view. We, and also Baker, do not find in Kolatchev and Mann Kopsch preparations a consistent blackening of homogeneous spheres, such as Hall figures. This author unfortunately gives no drawings of intravitaly stained *Euglenae*, and his figures of fixed preparations show a smaller number of 'osmiophilic globules' (vacuome) than we find of neutral red bodies in vitally stained organisms, or of osmiophilic rings in subsequently fixed *Euglenae*.

We have also found that the 'vacuome', or at least the contents of the 'vacuome', can be most easily demonstrated by methods recognized as unfavourable for the study of the Golgi apparatus of metazoon cells—i.e. Schaudinn's fixative followed by staining with methylene blue or other dyes. The fact that the bodies stain intravitaly with neutral red is, as Baker also pointed out, not evidence for, but against, their identity with true Golgi bodies.

Furthermore the neutral red stainable bodies of *Euglena* are denser than the cytoplasm and move to the centrifugal pole

on centrifuging, whilst the Golgi apparatus in all metazoon tissues so far examined have been shown to be of much less dense nature than the surrounding cytoplasm (Beams and King, 1934; Beams, Muliyl, and Gatenby, 1934). We realize, however, that the comparison of the relative specific gravity of materials in



TEXT-FIG. 1.

Photomicrograph. Mann Kopsch method, bleached. Periphery of reservoir blackened.

such widely separated groups of organisms cannot be taken as very convincing evidence.

Brown (1930) and Baker (1933) appear to agree in the identification of the Golgi bodies, and show them as a fairly small number of ring-like bodies scattered in the cytoplasm; though according to the former author they are most abundant near the nucleus. This author also states that they 'stain as spheres with black borders and clear centres. These ovoid bodies often show up as crescents when the material is poorly impregnated with osmic acid.' He also compares them to the osmiophilic platelets of plant cells (Bowen, 1928; Patten, Scott, and Gatenby, 1928).

The rings, spheres, and crescents seen by us in some preparations are, at first sight, very like those figured by Brown and Baker; but the structures shown by us are the periphery of the

neutral-red bodies, i.e. of the 'vacuome', and Baker has quite clearly figured 'vacuome' and Golgi bodies as distinct entities side by side in the same organism. Also the appearance of rings and crescents, as distinct from homogeneous spherules, seems in our preparations to be dependent on previous staining of the organisms with neutral red. We therefore think we have not figured anything comparable to the Golgi bodies of Brown and Baker. The latter mentions the irregularity and uncertainty of the appearance of these bodies in preparations made by the classic Golgi apparatus methods. We have, indeed, sometimes seen bodies that might be similar to those of Baker, but we were never certain that they were not artifacts.

The bodies which surround or form the reservoir (probably small contractile vacuoles) similar to those described by Sigot (1930), resemble Golgi material in one way, in that they resist bleaching very effectively. It is, however, well known that osmic acid is readily reduced in the walls of most vacuoles within cells, and that this blackening is often resistant to bleaching. Then, too, this interpretation is of little value in the identification of the Golgi material of other free-living flagellates; for instance, of the Euglenoid form, *Menoidium*, in which no blackening of the basal portion of the gullet was found. It is possible that the blackening of the peri-reservoir globules might be brought into line with the work of Duboscq and Grassé (1932) on the Golgi apparatus nature of the parabasal bodies of flagellates, but it seems more likely that the blackened structures of *Euglena* are vacuolar in nature.

In our opinion the identity of the Golgi material of Euglenoid flagellates is still uncertain, and cannot be profitably discussed until more is known and a comparison can be made with related forms.

#### IV. OBSERVATIONS ON *MENOIDIUM* SP.

We give a short account of this organism because in it there is frequently an interesting natural stratification to be seen (fig. 19, Pl. 32).

The large paramylum bodies are frequently, though not always, confined to the anterior one-half or two-thirds of the

organism; the use of intravital neutral red shows very small globules of a bright red colour also generally restricted to the same area as the paramylum. Behind this region there is a clear space which in fixed preparations is shown to contain the nucleus. Here the slightly spiral thickenings of the periplast can be most easily seen. In such forms as those mentioned above with paramylum in front, the posterior end of the organism is filled with small refractile bodies whose nature is undetermined (fig. 19 x., Pl. 32). When the organism is exposed to osmic vapour after neutral-red staining, these posterior globules are the first structures to darken (fig. 21, Pl. 32), and often even have a reddish tinge as though the neutral red diffused from its original position and passed to this region. Longer exposure to osmic vapour may blacken or darken the true neutral red bodies (fig. 22, Pl. 32). In Kolatchev and Mann Kopsch preparations neither posterior globules nor neutral-red-stainable bodies are usually blackened, though after Mann Kopsch's method the former may be conspicuously darkened, and sometimes also the latter, irrespective of whether the organisms have been previously stained with neutral red or not.

After centrifuging the natural stratification may be altered, depending, as in *Euglena*, on the orientation of the organism to the direction of the centrifugal force. The interesting point is that, as in *Euglena*, paramylum and neutral-red bodies go together. This is particularly well seen when they are deposited laterally (fig. 20, Pl. 32). The methylene-blue method colours bodies, having exactly the same distribution as the neutral-red-stainable bodies, blue to purplish colour, but has no effect on any of the other constituents (figs. 23 and 24, Pl. 32).

Hall (1931) homologized the 'vacuome' of *Menoidium incurvum* with the Golgi apparatus. He gives figures showing a small number of black 'osmiophilic globules' scattered quite irregularly through the cytoplasm.

#### V. *CHILOMONAS PARAMECIUM*.

Some preliminary experiments were carried out on this species. Observations closely resemble those made on the

Euglenoid forms but, owing to the very small size of this organism and to the lack of chloroplasts, effects are not so conspicuous.

After centrifuging, the starch grains behave as did the paramylum of *Euglena* and *Menoidium*, and are forced to one part of the organism (the centrifugal side). There is, however, so much starch that the stratification is not very clear. The neutral-red-stained bodies, which are scattered throughout the living control organism, become confined to the same region as the starch after centrifuging. Methylene-blue staining appears to indicate that the contents of these vacuoles corresponds to volutin. The presence of the latter substance in *Chilomonas* was shown a long time ago by Awerinzew (1907), though the granules in his figure do not appear to be nearly so abundant as in our preparations. Hall (1930) stained *Chilomonas* intravitaly with neutral red, and homologized the 'vacuome' so stained with the Golgi bodies. We have not noticed that impregnation with osmic acid blackens the contractile vacuole of *Chilomonas* as was shown by Nassanov (1924), but possibly this vacuole requires longer impregnation than does the reservoir and surrounding structures of *Euglena*.

## VI. SUMMARY.

1. When the three species of free-living flagellates employed in this investigation are subjected to the high centrifugal force obtained by the use of the air-driven centrifuge, stratification of the cytoplasmic components and inclusions takes place.

2. This stratification is most noticeable in the chlorophyll-bearing *Euglena*. The chloroplasts form a belt having on the centrifugal side paramylum and neutral-red bodies, while the clear cytoplasm containing small spherical bodies, probably mitochondria, is at the centripetal pole.

3. This stratification is a temporary process. Complete redistribution of the parts can take place. The orientation of the stratification is not dependent on the morphological polarity of the organism. The heaviest components may occupy the anterior, posterior, or lateral part of the organism.

4. There is no evidence that the bodies which stain intra-

vitality with neutral red are homologous with the Golgi bodies of the metazoa. On the contrary there is some new evidence to support the findings of Baker (1933) that these bodies stainable with neutral red give a metachromatic reaction with Meyer's methylene-blue method, and are therefore probably identical with volutin. We have reached no satisfactory conclusion regarding what structures represent the Golgi apparatus. The theories put forward by other observers are briefly discussed.

5. Fixatives containing osmic acid show spherical bodies close to the periphery of the organism. They are not moved by the centrifugal force.

6. Short notes are given on the effect of the ultra-centrifuge on *Menoidium* sp. and on *Chilomonas paramecium*. As in *Euglena* the heaviest materials in *Menoidium* are the paramylum and bodies stainable with neutral red. In *Chilomonas* starch grains and neutral-red-stainable bodies are displaced to the centrifugal pole. In control specimens of *Menoidium* there is sometimes a natural stratification to be observed—the paramylum and neutral-red bodies being gathered together usually at the anterior end of the organism.

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## DESCRIPTION OF PLATES 30, 31, AND 32.

Figs. 1-18, *Euglena* sp.; figs. 19-24, *Menoidium* sp.

All figures drawn with the aid of a camera lucida. Final magnification, 1600.

## LETTERING.

N.R., neutral-red bodies of living organisms and 'vacuome' of fixed preparations; c., chloroplasts; M., probable mitochondria; P., paramylum grains; s., stigma; x., peripheral bodies; y., posterior bodies of *Menoidium*.

## PLATE 30, figs. 1-8.

Fig. 1.—Living *Euglena*, stained with neutral red; uncentrifuged.

Figs. 2-6.—As for fig. 1, but all centrifuged. Stratification of the components has taken place in all cases, though not so marked in fig. 5; notice also that the orientation of the stratification bears no relation to the morphological polarity of the organism. Stigma fragmented in fig. 4. Mitochondria present in figs. 2 and 3.

Fig. 7.—Uncentrifuged. Kolatchev method. Dark spherules towards posterior end are the 'brown bodies'.

Fig. 8.—Centrifuged. Kolatchev method. Notice dispersal of peripheral bodies (x.) and mitochondria.

## PLATE 31, figs. 9-15.

Figs. 9 and 10.—Centrifuged. Kolatchev method. No mitochondria visible.

Fig. 11.—Organisms stained intravitaly with neutral red, centrifuged and then fixed by Mann Kopsch's method. Mitochondria abundant. Exterior of a few paramylum bodies darkened. Reservoir and surrounding structures intensely black.

Fig. 12.—Treated similarly to organism shown in fig. 11. Bodies stainable with neutral red (N.R.) show as clear spherules confined to centrifugal pole, while peripheral bodies (x.) are unaffected by the centrifuging.

Fig. 13.—Treatment as for figs. 11 and 12. 'Vacuome' in form of rings and crescents.

Fig. 14.—Organism stained vitally with neutral red (not centrifuged) and exposed to osmic vapour for 4 days. Bodies stainable with neutral red as for fig. 13, except that they are not confined to one pole.

Fig. 15.—As for fig. 14, but centrifuged. N.R. bodies confined to centrifugal pole.

## PLATE 32, figs. 16-24.

Fig. 16.—Uncentrifuged organism. Mann Kopsch preparation—bleached. Only reservoir remains blackened. Outline of chloroplasts and nucleus visible.

Figs. 17 and 18.—Centrifuged. Fixed Schaudinn's fluid stained Meyer's methylene-blue method for volutin. Notice chloroplast band in fig. 17.

Fig. 19.—Living *Menoidium*, stained with neutral red. Uncentrifuged.

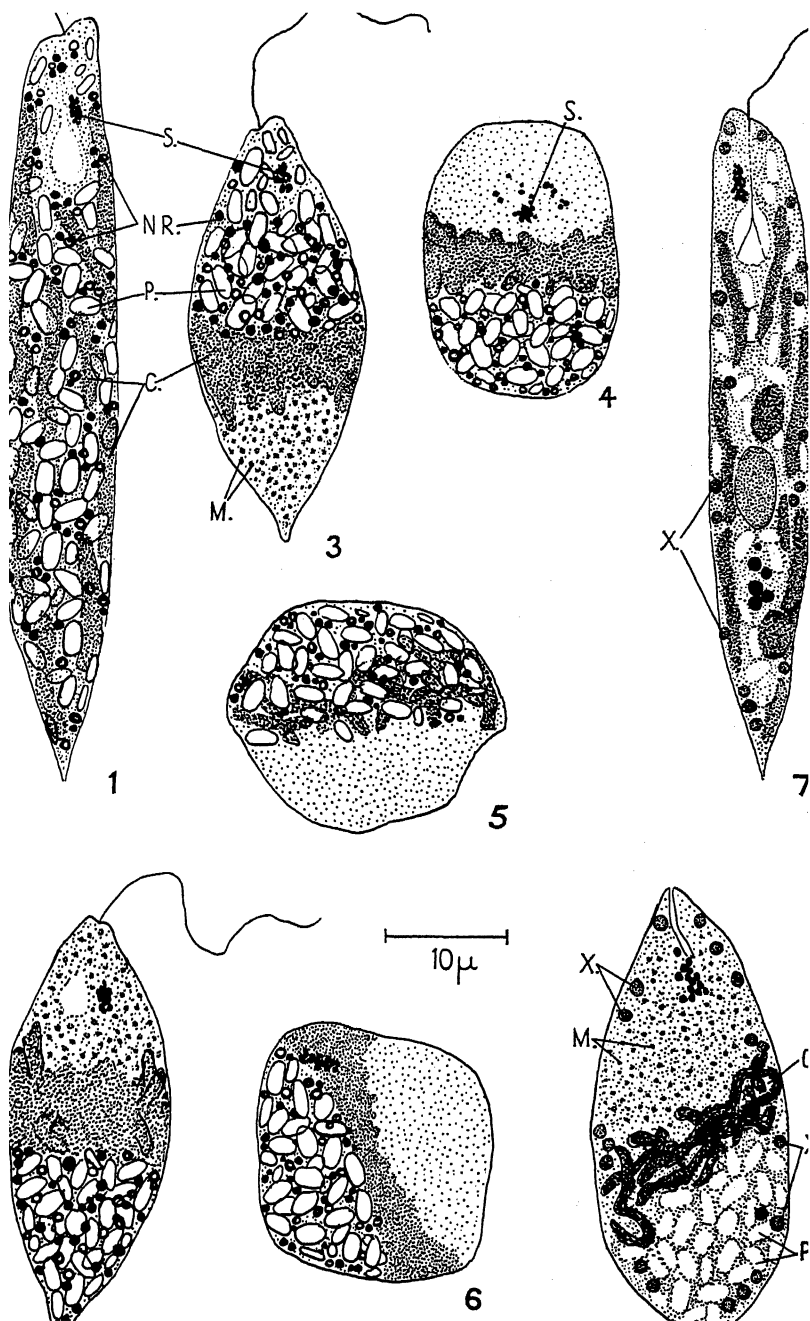
Fig. 20.—Do. but centrifuged—notice lateral position of paramylum grains and neutral-red bodies, though they often remain as in fig. 19.

Fig. 21.—Organism vitally stained with neutral red, then exposed to osmic vapour for 48 hours. No staining of neutral-red bodies but posterior spherules darkened.

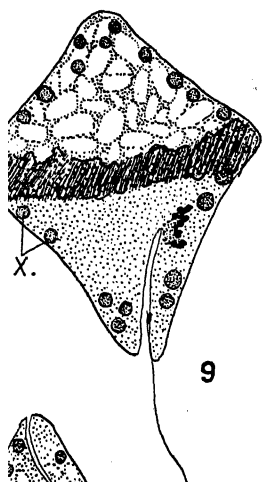
Fig. 22.—Method as for fig. 21, but exposure continued up till 60 hours. Both figs. 21 and 22 might equally well have been drawn from some of the material similarly fixed but not previously stained with neutral red, or from Mann Kopsch preparations whether following such intravital staining or not.

Figs. 23 and 24.—Organisms fixed Schaudinn's fluid and stained Meyer's methylene-blue method. Fig. 23 uncentrifuged. Fig. 24 centrifuged.









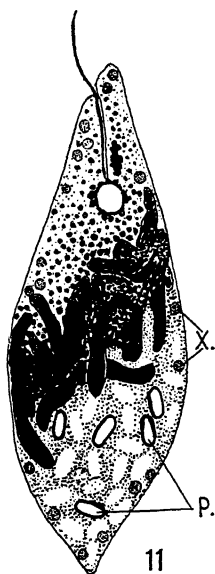
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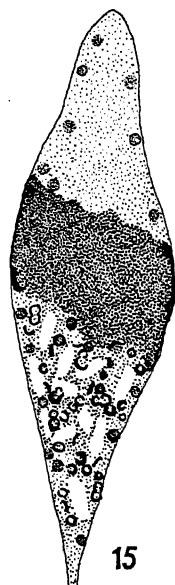


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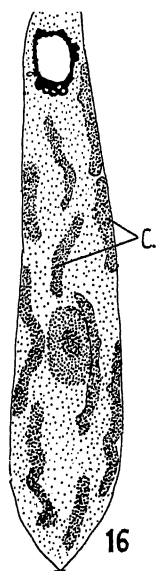


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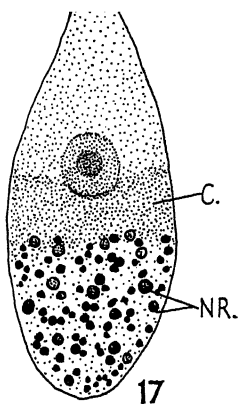


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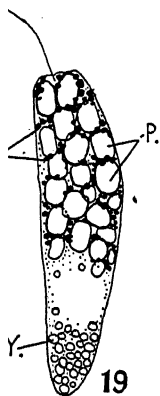
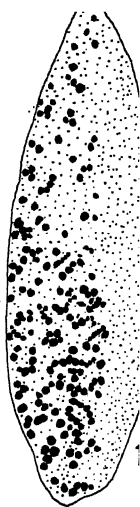




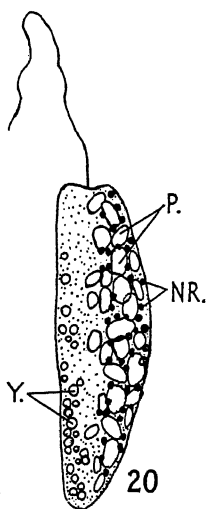
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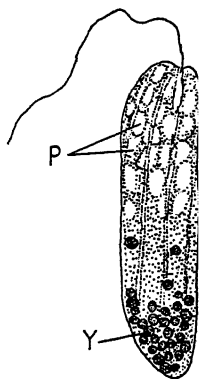
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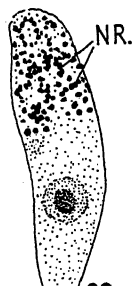
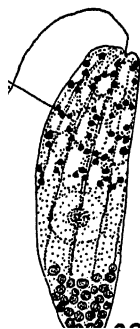


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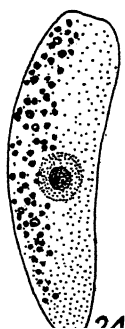


Y

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NR.



24





# A Study of the Histology of the Pituitary Gland of the Skate.

By

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University College, London.

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With Plates 33 and 34 and 5 Text-figures.

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## I. INTRODUCTION.

OUR knowledge of the functions of the pituitary gland, both in mammals and amphibia, has been considerably advanced by recent research, and, at the same time, attention has been directed to the comparative study of the gland throughout the vertebrates. One of the problems of especial interest is the correlation between histological structure and results obtained by the removal of different parts of the gland or by the injection of different extracts. In this paper the hypophysis of the skate has been studied from this point of view. The structure of the Selachian pituitary has already been to some extent described by other writers (Stendell, 1914; De Beer, 1925; Pokorny, 1926), but without reference to experimental work and without the application of the more delicate and special methods now used for the study of the histology of the gland in higher vertebrates. Some of these methods have been applied here and the results considered in relation to experimental work.

## II. MATERIAL AND METHODS.

Forty-six young and adult specimens of *Raia maculata* Mont., *Raia clavata* L., and *Raia brachyura* Laf. were used. The living animals were taken into the laboratory and a portion of the brain with pituitary intact rapidly removed and dropped into fixative. To do this, transverse incisions were made from the dorsal surface down through the cranium into the mouth, one at the level of the spiracle and the second at

that of the anterior margin of the eye. This length of cranium was completely isolated by lateral longitudinal cuts and the whole removed from the animal; by turning this upside down, the pars ventralis could be detached from the floor and the whole piece of brain, complete with pituitary, dropped into fixative. In this way, not more than five minutes elapsed between the making of the first cut and the beginning of fixation.

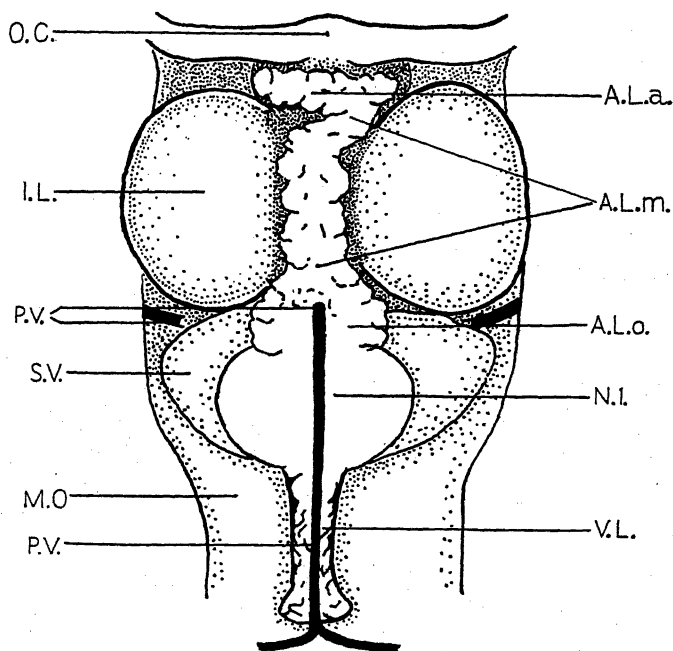
The following fixatives were used: Bouin, Carnoy, Da Fano, Heidenhain's Suza, Gilson, formol bichromate, of which Suza gave the least shrinkage. The material was embedded in paraffin wax and serial sections cut in transverse, horizontal, and sagittal planes. Sections were stained in Heidenhain's Azan, Giemsa, Ehrlich's haematoxylin and eosin or Biebrich scarlet, Hastings-Romanowsky, Heidenhain's haematoxylin, Mallory's triple stain, Weigert's iron haematoxylin, and by the iodine-leucobase technique of Spaul and Howes (1930).

### III. MORPHOLOGY OF THE GLAND.

The morphology of the gland is well known, but a brief description is necessary for purposes of reference. As seen *in situ*, the gland exhibits a slender anterior region (pars anterior of De Beer, Hauptlappen of Stendell) lying in the mid-line between the lobi inferiores and extending back from the optic chiasma to their posterior limit: at this point a process, apparently continuous with that just described, leaves the gland at an angle of about  $120^\circ$  with the pars anterior and passes ventrally to the floor of the cranial cavity—this is the ventral lobe. At the angle it is attached dorsally to the ventral surface of a spherical portion of the gland lying at the posterior end of the pars anterior, the neuro-intermediate lobe. Above this lies the dark-red saccus vasculosus with large paired flaccid lateral extensions (Text-fig. 1).

Macroscopically the gland appears to consist of a skein of continuous tubules, but it was found to be impossible to unravel these by dissection.

An examination of fixed glands from animals of various sizes, prior to embedding, revealed certain well marked variations in size and shape. With increase in size of the animal, there



TEXT-FIG. 1.

Diagram of the skate pituitary seen from below. The ventral lobe is turned backwards and dorsally.

#### LETTERING FOR TEXT-FIGS. 1-4.

*A.L.*, anterior lobe; *A.L.a.*, anterior basiphil region of anterior lobe; *A.L.m.*, middle faintly basiphil region of anterior lobe; *A.L.o.*, oxyphil region of anterior lobe; *B.*, basiphil cell; *B.V.*, blood-vessel; *C.M.*, colloidal material in hypophyseal cavity; *H.C.*, hypophyseal cavity; *I.L.*, inferior lobe; *M.O.*, medulla oblongata; *N.I.*, neuro-intermediate lobe; *O.*, oxyphil cell; *O.C.*, optic chiasma; *O.Ex.*, oxyphil cell extruding contents into blood-vessel; *P.I.*, pars intermedia; *P.N.*, pars nervosa; *P.V.*, pituitary vein; *S.V.*, saccus vasculosus; *S.V.c.*, cavity of saccus vasculosus; *3rd V.*, third ventricle; *V.L.*, ventral lobe.

was (a) an increase in length of the pars anterior, both absolutely and relatively, resulting in the bending of the anterior end away from the mid-line towards the right and the growth of a short transverse portion, so that the tip of the gland was frequently

directed backwards; (b) an increase in size of that region of the pars anterior immediately in front of the neuro-intermediate lobe, both in thickness and width. These variations could be correlated with the size of the animal, irrespective of species and sexual maturity. Incidentally, the cells in both these regions show characteristic staining reactions.

#### IV. HISTOLOGY.

##### Pars Anterior.

Sections showed that in young specimens this region is roughly tubular with an epithelium five to ten cells thick surrounding the central longitudinal hypophyseal cavity. On the ventral surface towards the anterior end, this cavity was extended into diverticula formed by growth and folding of the epithelium. This folding increased enormously on the ventral surface until, in older specimens, the originally large cavity was reduced to a narrow canal running below the dorsal epithelium. The use of the graphical method with serial sections showed that the diverticula remained in continuity with the original cavity and were not penetrated by blood-vessels. The external surfaces of the folds were usually in contact with connective tissue enclosing capillaries arising from the cerebral artery. The sheath of connective tissue was interrupted at intervals so that cells came into direct contact with the vessel and could be found extruding their granular contents directly into the blood-stream (Text-fig. 2). The epithelium thus presents one surface to the hypophyseal cavity while the other is in mainly indirect contact with a blood-vessel.

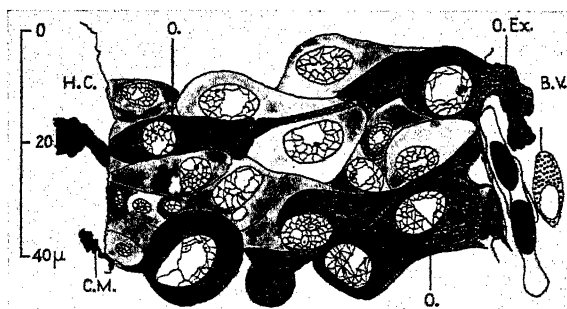
The cells forming this epithelium are typically oval in section, with one end drawn out into a thinnish process, so orientated as to lie with their long axes perpendicular to the surface. Three types could be distinguished: (a) large cells about  $40\mu$  long and  $20\mu$  thick, with large nuclei; (b) intermediate; and (c) small cells (Text-figs. 2 and 4). The large cells, which predominated in all regions of the anterior lobe, were usually, in company with the other types, faintly basophil in reaction with Mallory's stain. In the anterior and posterior regions many of them were strongly chromophil.

The nuclei of all the cells appeared to be in the resting state and no trace of mitosis was found even in young glands.

Finely granular blue-staining material was found both in capillaries and hypophyseal cavity of this region.

The staining reaction of the cells of the pars anterior varied according to the methods applied:

1. Stains of the Giemsa type gave a different picture from that



TEXT-FIG. 2.

Oxyphil region of female *Raia clavata*. An oxyphil cell extruding its contents into a blood-vessel is seen at the top right-hand corner.

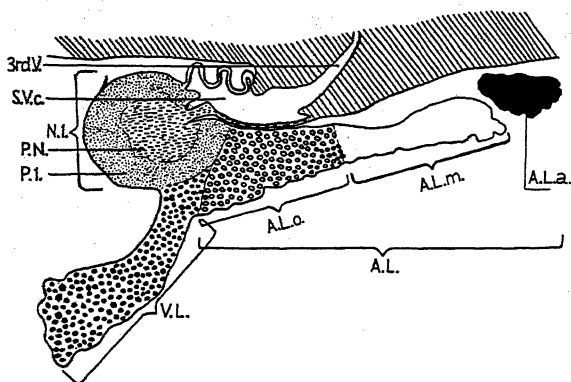
obtained with Mallory's stain, using similarly fixed material. By the former methods, results similar to those obtained by Stendell (1914) and De Beer (1926) were obtained, namely an orientation of oxyphil cells towards the blood-vessels and basiphil cells towards the hypophyseal cavity.

2. With Mallory the majority of cells stained pale blue, but cells giving three types of chromaphil reaction were found with the following distribution:

(a) At the anterior end, especially towards the ventral surface, considerable numbers of deep wine-coloured cells were found. These differed from their neighbours only in staining reaction, the latter being pale blue. Near the hypophyseal cavity the chromaphil cells were clear cut in outline and closely packed with fine granules, but in the vicinity of the blood-vessels—frequently found to contain granular masses of basiphil material

—they became less discrete and their granulation coarser and more diffuse. In preparations stained with iron haematoxylin, cells corresponding to these were found to retain the dye long after neighbouring cells were completely decolorized.

(b) The cells of the middle region were almost exclusively chromophobe, closely resembling those of the anterior region and like them staining a pale blue. Rarely a deep-blue chromophil cell was found amongst them. This region elongates with



TEXT-FIG. 3.

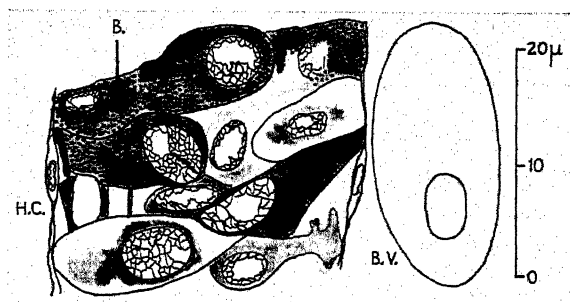
Diagrammatic median sagittal section of skate pituitary.

increase in size of the gland, being short in small specimens and forming nearly half the gland in older ones.

(c) In the posterior end of the pars anterior the majority of cells were of the faintly basophil type just described, but numerous chromophil cells were found scattered among them, the majority oxyphil and a few basiphil. The oxyphil cells were scattered throughout this region and stained bright scarlet with Mallory (fig. 1, Pl. 33). Those near the hypophyseal cavity were discrete and filled with fine granules, but towards the capillaries the granules became larger and less numerous and the outline of the cell less clearly defined. Frequently cells of this type appeared to be extruding their contents into the blood-vessels which in this region contained red granular material in addition to the blue matter described above (Text-fig. 2). Among these

oxyphil cells were found basophil cells with deep blue-staining coarse reticular protoplasm. These were usually smaller than the oxyphil cells and frequently occurred in their neighbourhood although no significant relationship appeared to exist between them. Chromaphil cells staining deeply with iron haematoxylin were found in the corresponding region of the pituitary of *Squatina angelus* by Pokorny (1926).

The application of the iodine-leucobase technique of Spaul and Howes (1930) to the gland, revealed that cells corresponding



TEXT-FIG. 4.

Section of anterior portion of pars anterior of female *Raia maculata* showing chromaphil cells.

in shape, size, and distribution to the oxyphil cells possess an affinity for iodine similar to that shown by the oxyphil cells of the ox pituitary. Hence it is possible that there is something in common between the chemical constitution of these cells in higher and in lower vertebrates.

#### Inferior Lobe.

In sagittal sections of the whole gland the epithelium of the pars anterior has the appearance of being continuous with that of the ventral lobe. At its posterior end the epithelium seems to bend ventrally, continue along the lower surface of the neuro-intermediate lobe, and pass into the ventral lobe where it becomes free from the latter. At the dorsal surface only does



there appear to be continuity between the pars anterior and the neuro-intermediate lobe.

There was considerable variation in the degree to which the antero-dorsal region of the ventral lobe was attached to the neuro-intermediate lobe. In some cases the former was only attached to the extreme anterior end, in others it lay along the whole of the ventral surface. In section the two were easily distinguished by the marked differences in structure and staining reaction. In many cases the tissues of the ventral lobe came into direct contact with those of the neuro-intermediate lobe, but in the mid-line a longitudinal blood-vessel, a branch of the internal carotid artery, was usually found running along the centre of the lobe. The cells of that part of the inferior lobe in contact with the neuro-intermediate lobe were large and faintly basiphil. Apart from being slightly larger, they closely resembled the cells of the middle region of the pars anterior.

In the free, descending part of the lobe the character of the cells gradually changed. Those at the outside and bordering the blood-vessel had a dense cytoplasm, but a zone of apparently degenerating cells was commonly found at the centre of the epithelium, and in this zone lay larger cells, containing loosely packed coarse oxyphil granules (De Beer, 1926). These seemed to occupy positions as remote as possible from both surface and blood-vessels. Cells containing deeply basiphil granules were also found in the same region; rarely they were small and isolated but more often they formed a cap round the bigger oxyphil cells (fig. 5, Pl. 34).

In large specimens the cells situated towards the postero-ventral part of the lobe occurred in nests surrounded by vascular connective tissue trabeculae. Near the connective tissue the cells were distinct and individual, but towards the centre there was a mass of apparently degenerate cellular material consisting of coarse, faintly basiphil granules containing a few indistinct nuclei. In this tissue oxyphil and basiphil cells were found. They had large, widely spaced granules with chromaphobe cytoplasm between them. Finally, occasional dividing cells were found in this region.

### Neuro-intermediate Lobe.

This lobe is so called because it contains an antero-dorsal mass of neuroglial fibres surrounded by a mass of glandular tissue (Stendell, 1914; De Beer, 1926). The fibres of the pars nervosa were apparently in continuity with others originating from the floor of the third ventricle, which passed back as a band about 1 mm. wide under the epithelium of the ventral wall of the saccus vasculosus and entered the lobe at its antero-dorsal surface (fig. 3, Pl. 34). According to Dammerman (1912) this band contains nerve-fibres which connect sense organs in the saccus with their ganglia in the brain. Since the saccus vasculosus is an outgrowth of the infundibulum, this latter, unlike that of the higher vertebrates, is not tubular in the skate, but retains its nervous connexion with the brain on its anterior face only. The pars nervosa is embedded in the glandular tissue of the pars intermedia into which it merges without any definite line of demarcation.

It consists predominantly of large and small neuroglial cells with large nuclei surrounded by a thin layer of cytoplasm which has long, branching processes. Among these cells isolated ovoid cells with granular oxyphil cytoplasm were found. Occasionally masses of a clear colloid-like material, staining yellow with picric acid, pink with eosin, and red with Mallory, occurred.

The neuroglia is largely concentrated at the anterior of the lobe and posteriorly and peripherally the gland becomes increasingly glandular in appearance (fig. 4, Pl. 34). This region, usually called the pars intermedia, is very vascular and somewhat sketchily divided into tubule-like regions, separated by connective tissue trabeculae. The 'lumina' of the tubules are filled with connective tissue, apparently neuroglia continuous with the pars nervosa. Occasionally, a space containing basophil colloid matter is found in the trabeculae (fig. 5, Pl. 34). The cells are columnar, about  $40\mu$  long and 6 to  $10\mu$  wide, with their bases resting on the connective tissue. The free ends were roughly stellate in section and contained a spherical nucleus. With all the staining methods applied these cells showed an oxyphil reaction, as described by Stendell. Their cytoplasm was granular and exhibited slight differences in staining reaction.

With Heidenhain's haematoxylin they retained the stain in very variable degrees, some being rapidly, others only tardily, differentiated, with a complete series of intermediate stages. No morphological differences between these cells were found. They all contained clear droplets staining specifically with picric acid. A few smaller cells with clear cytoplasm occurred among these larger ones.

### The Saccus Vasculosus.

This organ is not, according to Dammerman, physiologically associated with the pituitary, but is a sense organ concerned with the regulation of the pressure of the cerebro-spinal fluid, and contains groups of sense cells connected by nerve-fibres to ganglia—the ganglia sacci vasculosi—lying on either side of the infundibulum where it joins the brain. Histological findings confirmed those of Dammerman except in one particular. He identifies the anterior median portion of the saccus as the infundibulum proper, with a cubical epithelium continuous with the ependyma of the third ventricle. Beneath this, a strand of nervous tissue carries fibres to the sense organs of the saccus and neuroglia fibres to the pars nervosa; laterally and posteriorly the infundibulum almost disappears owing to the enormous outgrowths of the saccus, and is represented solely by the epithelium of the latter. He states that at all points there is a continuous epithelium between the cavity of the saccus and the hypophysis. However, in sagittal sections of that region where the neuroglia enters the neuro-intermediate lobe, a thin, horizontal strip of tissue of the pars nervosa was found directly abutting on the cavity. This corresponded to the region between the infundibulum proper and the saccus. In relation to this it is of interest that Spaul (unpublished) found evidence of the presence of the melanophore stimulant in extracts of the saccus vasculosus. On the other hand, no sign of colloid material was found in the cavity.

### V. DISCUSSION.

The value of the staining reaction of cells as an indication of the physiological functions has always been a subject of dispute, especially among workers on the pituitary gland

(Sterzi, Rossi, Stendell), and, in the present state of incomplete understanding of the chemical and physico-chemical processes involved in staining, it is perhaps inadvisable to infer too much from results so obtained by one method. Nevertheless, the Mallory method here applied has given consistent results with various fixatives—results which seem to permit of more definite correlation between the component cells of this gland and those in the pituitary of higher vertebrates than that obtained by other methods. The correlation of the distribution of the oxyphil cells with the activity of extracts in producing accelerated metamorphosis in amphibia from different regions in the ox pituitary (Spaul and Howes, 1930) supports this contention. Likewise, extracts of the anterior lobe of the skate, which contains only a very few oxyphil cells, induced slight acceleration of metamorphosis in tadpoles under favourable conditions (Spaul, unpublished). It may be that the stain is more sensitive to differences in cytoplasmic constitution than the others applied, and it is quite possible that others may be eventually described having an even greater degree of selectivity.

The fact that the oxyphil cells are confined to a region where growth continues into adult life suggests that they are of permanent physiological significance. Finally, the oxyphil cells show an iodine-leucobase reaction similar to that described for acidophil cells in the ox gland. On the other hand, the differences between the reactions of the cells to Mallory and to the other staining methods is not found in the pituitaries of higher forms.

As to the homology between the various regions of the skate pituitary and those of higher forms, it is only possible to suggest relationships. While a large amount of work has been done on the early development of the gland and its adult form (Balfour, 1878; Haller, 1898; Gentes, 1908; Woerdeman, 1914; De Beer, 1926), there remains a hiatus in which no work has been done linking up the early histogenesis with the adult structure. Stendell (1914, p. 153) gives a scheme homologizing the different parts of the pituitary in various vertebrates, and De Beer (1926, p. 101) an improved but essentially similar scheme for the evolution of the gland. Both authors homologize the pars

anterior of the skate with the pars anterior of mammals, and the neuro-intermediate lobe with the partes nervosa and intermedia. There is no trace of the ventral lobe in mammals. Woerdeman (1914, p. 254) gives a different scheme derived from the study of the early development of the gland. He divides the pituitary into four regions, of which only the posterior is derived from Rathke's pouch proper, the remainder originating from a further ingrowth of buccal epithelium. The gland thus consists of an anterior 'Vorraum', a 'Mittelraum' from the lower part of which lobi laterales arise, and a posterior Rathke's pouch. From Woerdeman's figures the corresponding regions would be:

<i>Region of Gland.</i>	<i>Adult Mammal.</i>	<i>Adult Skate.</i>
Pars anterior	Rathke's pouch + 'Mittelraum'	'Vorraum' + 'Mittelraum' + Rathke's pouch
Pars intermedia	Rathke's pouch	Rathke's pouch
Pars tuberalis	'Vorraum' possibly + lobi laterales	Not identified
Pars ventralis	Not identified	Lobi laterales

It is therefore possible that the pars intermedia is homologous in both classes although different in structure, being derived from that part of Rathke's pouch which comes into contact with the infundibulum.

Again, the fact that chromaphil cells of a type similar to those found in mammals occur only at the posterior end of the pars anterior of the skate makes it possible that such cells are in both cases ultimately derived from Rathke's pouch. On the other hand, the occurrence of cells at the very anterior end of the skate pituitary similar in staining reaction to those of the pars tuberalis of other vertebrates is clearly reminiscent of the condition found in many Urodeles. Here the pars tuberalis consists of paired processes of the anterior end of the pars anterior (Atwell, 1921; Sumi, 1926), which in some species become detached to form separate epithelial plaques, e.g. in *Diemictylus pyrrhogaster* (Sumi, 1926), leading to the condition found in *Anura* and thence to that found in higher vertebrates. If this be the case, then the middle portion of the

pars anterior of the skate would be homologous with the 'Mittelraum' of Woerdeman (1915), which quite possibly corresponds to the basiphil area in the pituitary of the ox described by Spaul and Howes (1930).

In the adult skate gland the pars ventralis appears to be continuous with the pars anterior but it is structurally entirely different from and actually independent of the pars intermedia. This would agree with Woerdeman's scheme that it is derived from the lobi laterales.

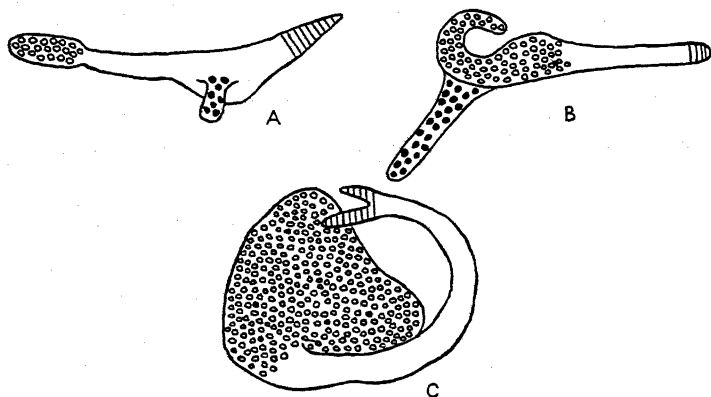
Thus, in so far as it is possible from the study of adult material only, to homologize the different parts of the skate pituitary with that of the mammal, the following scheme is suggested:

<i>Embryonic structure, Woerdeman.</i>	<i>Skate.</i>	<i>Mammal.</i>
'Vorraum'	Anterior tip of pars anterior ('Vorraum' only)	Pars tuberalis
'Mittelraum'	Middle faintly basiphil region	Basiphil region of pars anterior
Rathke's pouch	Posterior oxyphil region of pars anterior	Oxyphil region of pars anterior
Rathke's pouch	In contact with infundibulum becomes pars intermedia	In contact with infundibulum becomes pars intermedia
Lobi laterales	Pars ventralis	May form part of pars tuberalis

My sincere thanks are due to Professor E. A. Spaul for his suggestions and advice during the early part of this work and for assistance in collecting material; to the staff of the Marine Biological Association, Plymouth, for their help and courtesy; and to Mr. G. A. Steven, who kindly identified the species of the fish used. The material was collected while occupying the University of London table at the Marine Biological Station, Plymouth, and the work begun at Birkbeck College, London. I am indebted to Professor D. M. S. Watson and to Professor L. T. Hogben for reading the typescript and for making several valuable suggestions. My acknowledgements are also due to Mr. J. R. Thomas of this department, who took the photographs.

## VI. SUMMARY.

1. The structure of the pituitary of the adult skate is described.
2. This gland shows two distinct regions of growth which can be correlated with increase of size of the animal.



TEXT-FIG. 5.

Diagram showing homologies between skate and mammalian pituitaries. Anterior end to right. A. Embryonic skate, from Woerdeman. B. Adult skate. C. Late embryonic mammal, slightly modified from Woerdeman. Circles, Rathke's pouch and its derivatives; dots, lobi laterales; lines, 'Vorraum'; white, 'Mittelraum'.

3. The pars anterior can be subdivided into three regions differing by the staining reactions of their constituent cells: (a) an anterior region where deep-purple chromophil cells are found; (b) a middle, where they are faintly basiphil; and (c) a posterior, where they are mainly acidophil.

4. It is suggested that these regions are homologous with the pars tuberalis, basiphil, and oxyphil areas respectively of the pars anterior of the mammalian pituitary.

5. The oxyphil cells show an iodine-leucobase reaction similar to that given by the oxyphil cells of the ox pituitary.

6. The ventral lobe is a completely separate structure from the pars intermedia, although it may run along the ventral surface of the latter for some distance.

7. The histology of the neuro-intermediate lobe is described.

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## EXPLANATION OF PLATES 33 AND 34.

## LETTERING.

B., basiphil cell; B.V., blood-vessel; H.C., hypophyseal cavity; N.S., strand of nervous tissue containing nerve-fibres to sense organs of saccus vasculosus and neuroglia fibres to pars nervosa; O., oxyphil cell; P.A., pars anterior; P.I., pars intermedia; P.N., pars nervosa; P.V., ventral lobe; S.V., saccus vasculosus.

## PLATE 33.

Fig. 1.—Transverse section of posterior end of pars anterior. Female *Raia clavata*. Suza. Mallory.  $\times 200$ . The oxyphil cells appear as black dots.

Fig. 2.—Transverse section of the ventral lobe. Male *Raia clavata*. Suza. Mallory.  $\times 270$ . The large granular oxyphil cells with associated basiphil cells are visible at the centre of the photograph.

## PLATE 34.

Fig. 3.—Longitudinal section of the region where the pars anterior meets the neuro-intermediate lobe. Female *Raia maculata*. Suza. Heidenhain’s iron haematoxylin.  $\times 30$ .

Fig. 4.—Longitudinal section of the neuro-intermediate lobe. Female *Raia brachyura*. Suza. Mallory.  $\times 30$ .

Fig. 5.—Longitudinal section of pars intermedia. Female *Raia maculata*. Suza. Mallory.  $\times 330$ .





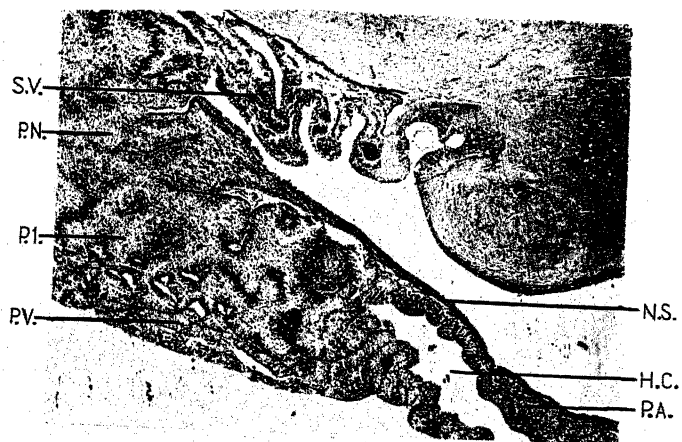


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# The Internal Anatomy of *Baccalaureus* with a Description of a New Species.

By

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With Plates 35 to 40 and 9 Text-figures.

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THE genus *Baccalaureus* was formed by Broch in 1929 for Ascothoracica from the coast of Japan. He placed this new genus in the Lauridae, and described one species, *B. japonicus*. The internal anatomy of this species was described two years later by Yosii, and in 1934 a second species, *B. maldivensis*, was added to the genus.

Through the kindness of Captain A. K. Totton of the British Museum, whose help in this connexion I must gratefully acknowledge, I have been able to examine specimens of *Baccalaureus*, recently sent to the Museum by Professor F. J.

Meggitt of the University College, Rangoon, within Zoanthid polyps of the genus *Palythoa*. These were collected at Maungmagan, near Tapoy, on the west coast of Burma.

These specimens closely resemble *B. maldivensis*, but show sufficient differences to warrant being placed in a separate, though closely allied species. The material was freshly preserved, and it has been possible to examine the internal anatomy in some detail. I have thus been able to complete my investigations started on *B. maldivensis* and also to amplify, and in some respects to modify, Yosii's work on *B. japonicus*.

### 1. LOCATION AND METHODS OF EXAMINATION.

Twenty-four specimens were available for examination. The larger polyps were heavily infested, four having two *Ascothoracica* within, and one polyp having as many as four. In the latter case there was considerable distortion of the upper part of the polyp. The specimens were all found within the enteric cavity of the Zoanthid, and in every case microscopic examination showed that the parasites were wholly enclosed within a layer of Zoanthid tissue. Yosii (10) states that *B. japonicus* forms galls on *Palythoa*, but does not say whether internally or externally.

Eighteen specimens were attached in the upper part of the enteric cavity, just below the stomodaeum, three mid-way, and one at the base. Two specimens were unattached, but a detailed examination failed to show that these differed in any way from the attached forms, so that it is probable that they had broken away accidentally.

Examination was carried out by three methods:

Serial sections of whole specimens.

Dissection of whole specimens.

Dissection, followed by section of the segmented body.

The first of these methods gave the most useful results. The second method was used for the investigation of special points, for example, the arrangement of the gut branches and yolk-glands, the development of the yolk-glands, the antennae, &c. The third method was unsatisfactory, as separation of the segmented body involved cutting away much of the mantle

wall laterally and dorsally and removing part of the adductor muscle, which ruined the anterior part of the segmented body.

Sections were cut at a uniform thickness of  $10\mu$ . They were stained by two methods: Mallory's triple stain and 1 per cent. acid fuchsin, and aqueous magenta and picro-indigo-carmin. The triple stain gave adequate differentiation despite fixation in formalin, but, apart from the chitinous structures, the differentiation with magenta and picro-indigo-carmin was poor.

## 2. THE EXTERNAL ANATOMY.

The following description of the anatomy, both external and internal, is given with respect to the morphological orientation of the segmented body, and not with respect to the animal as a whole.

Generally speaking, the external anatomy closely resembles that of *B. japonicus* and *B. maldivensis*, accounts of which have already appeared (Broch, 1; Yosii, 10; Pyefinch, 8), but it has been possible to amplify several details of the external structure in the present series of specimens.

### (a) The Chitinous Ridge.

This ridge (the 'chitinleiste' of Broch) has been observed on every species of *Baccalaureus*. As Yosii has pointed out, it is merely a local thickening of the chitinous body-wall, and appears distinct because the body-wall elsewhere is very thin. In the present species it runs along the side of the body, roughly half-way between the dorsal surface and the bases of the thoracic appendages. It runs back at this level until the fourth free thoracic segment; here it turns dorsally and flattens slightly. It runs obliquely over the first abdominal segment, finally crossing over to meet its fellow of the opposite side at the posterior border of this segment. Anteriorly, as in *B. japonicus*, it runs forward on to the antennules ('long horn-like processes'—Yosii) and forms part of the outer lateral wall of these.

It becomes less and less distinct and finally fades away towards their tip.



## (b) The Appendages.

### (i) The Antennules.

These appendages have been studied whole, in transverse and in longitudinal section. They arise from the upper part of the head, running forward parallel with each other for a short distance, then coil outwards in a loose spiral into the lumen of the lateral coils of the mantle. Their tips reach the outermost coil.

In transverse section (figs. 1, 2, Pl. 35) part of the outer lateral wall is seen to be thickened. This is the anterior prolongation of the chitinous ridge. The rest of the chitinous wall is thin and delicate, so that it usually appears crumpled in section. It bears numerous fine setae (fig. 2, Pl. 35). Within is an irregular epidermal layer, and the appendage is filled with a loose connective tissue, with large amoebocytes here and there. There are transverse muscle strands, and strong longitudinal muscles, which are part of the large adductor muscle and are attached to the base of the appendage (Text-fig. 1, *d*, *e*).

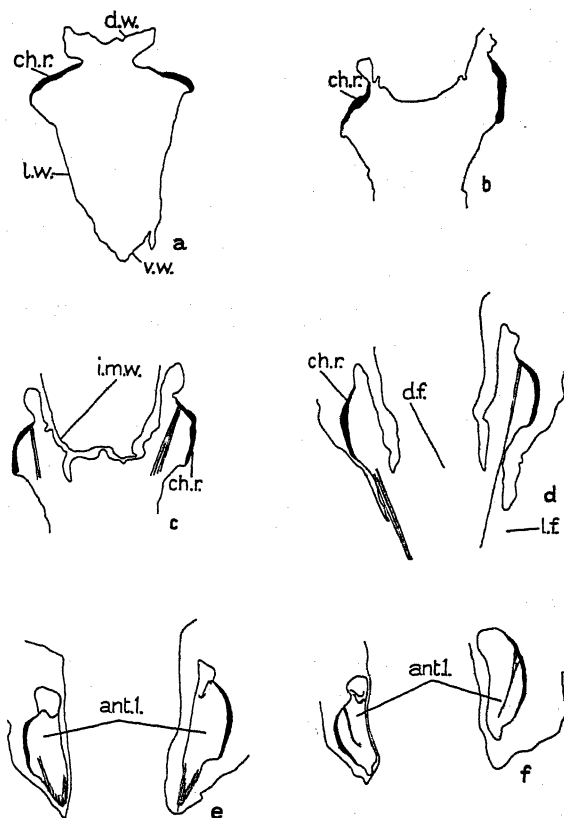
In some cases the internal connective tissue is denser than in others (cf. figs. 1 and 2, Pl. 35), but there is no evidence that this difference has any functional significance, indeed, the function of these appendages as a whole is difficult to envisage.

Yosii, in his description of *B. japonicus*, follows Broch and calls these appendages 'long horn-like processes'. In the female he applies the term antennules to a pair of appendages posterior to the adductor muscle and post-oral. This interpretation seems open to some doubt, especially as in the male the same name is given to an appendage which is pre-oral and anterior to the adductor, and which corresponds, in fact, to the appendages described above. From an examination of the present series of specimens it is suggested that the latter interpretation is more likely to be correct, and that the appendages termed antennule in the female of *B. japonicus* might be regarded as the first thoracic appendages.

### (ii) The Antennae.

In all previous accounts of the segmented body of *Bacca-laureus*, a description has been given of a structure termed

'blasenformige Anschwellung' (Broch, Yosii) or 'ovoid capsule'. It has been possible to dissect this 'capsule' away from the



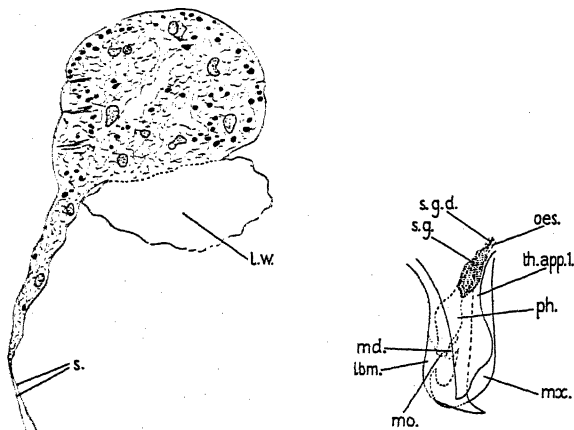
TEXT-FIG. 1.

Vertical sections of the segmented body-wall showing chitinous ridge, and origin of antennules. ( $\times 130$ .) *ant.l.*, antennule; *ch.r.*, chitinous ridge; *d.f.*, dorsal fusion of segmented body; *d.w.*, dorsal body-wall; *i.m.w.*, inner mantle-wall; *l.f.*, lateral fusion of segmented body; *l.w.*, lateral body-wall; *v.w.*, ventral body-wall.

segmented body. It is then seen that it is attached to the side of the head below the chitinous ridge (fig. 13, Pl. 36), and that anteriorly it has a limb-like projection running obliquely for-

wards and downwards over the head (Text-fig. 2). This projection ends in two fine setae. In section this 'capsule' is seen to be a flattened sac filled with a loose connective tissue (fig. 13, Pl. 36).

It seems probable that this capsular structure is an appendage, and from its relationships with the head and the anten-



TEXT-FIGS. 2-3.

Fig. 2.—Lateral view of left antenna, dissected away from body-wall. ( $\times 235$ .) *l.w.*, lateral body-wall; *s.*, setae.

Fig. 3.—Reconstruction of the oral cone. ( $\times 130$ .) *lbm.*, labrum; *md.*, mandible; *mo.*, mouth; *mx.*, maxilla; *oes.*, oesophagus; *ph.*, pharynx; *s.g.*, gastric gland; *s.g.d.*, duct of gastric gland; *th.app.1*, first thoracic appendage.

nules I would suggest, tentatively, that it is the antenna. Owing to the telescoping of the head and the anterior thoracic segments, and the partial degeneration of the head, the identity of this appendage cannot be determined with greater precision.

### (iii) The Oral Cone.

The oral cone is so buried beneath the adductor muscle and mantle-wall that it has been investigated only in section. A reconstruction is given in Text-fig. 3. It differs in the degree of development of its components from that of *B. japonicus*

and *B. maldivensis*. A comparison of the three species is given in Table I below:

TABLE I.

<i>Appendage.</i>	<i>B. japonicus.</i>	<i>B. maldivensis.</i>	<i>B. hexapus.</i>
Labrum. . .	+	+	+
Mandibles . .	0	0	0
Maxillulae . .	+	0	—
Maxillae . . .	+	+	+
First Thoracic App. = Antennule?	+	+	+

+ indicates present and large; 0 indicates present, but small; — indicates absent.

The whole cone is well developed, and strong retractor muscles are attached antero-laterally (fig. 10, *r.m.o.c.*, Pl. 36).

The labrum, which ensheaths the oral cone anteriorly, laterally, and to some extent posteriorly, has a thick outer wall and a thin inner wall (figs. 3–7, *lbm.*, Pl. 35). The space between these two walls is filled with connective tissue, and is traversed by well-developed muscle-bands. Attached to the inside of the lateral wall of the labrum, at the level of the tip of the mandible, is a row of strong setae which appear comb-like (figs. 4, 5, *c.s.*, Pl. 35). A transverse muscle-band is attached immediately below this row. Contraction of the muscle would draw the inner wall outwards and so draw the setae downwards and outwards, scraping them against the outer surface of the mandibles. There is also a small group of fine setae, on a dorsal projection of the mantle wall, which scrape against the outer surface of the labrum (fig. 5, *s.m.*, Pl. 35).

The mandibles consist of an outer finger-shaped part, and an inner plate (fig. 4, Pl. 35). The outer part has a thickened chitinous wall laterally and distally, but the inner plate is thin-walled. Muscles, running dorsally, are attached to the latter. Contraction of these would draw the outer parts in towards the mouth.

The maxillulae are entirely absent from the present series of specimens.

The maxillae are the largest of the mouth parts. In shape and internal structure they are practically identical with those

of *B. maldivensis*. The two maxillae are fused in the middle line except at their tip. These tips are pointed and are turned up towards the mouth (fig. 3, Pl. 35). There are a few fine setae on the outside of the maxilla which scrape against the inside of the labrum (fig. 6, Pl. 35). Two large retractor muscles are attached to the base of the maxillae and run dorsally, passing outside the oesophagus and circumoesophageal commissures, to the mantle-wall (fig. 4, Pl. 35; fig. 11, *r.m.mx.*, Pl. 36).

**The First Thoracic Appendages.**—These are closely connected with the oral cone, so that they may be considered here. They can be distinguished from the other mouth parts because their chitinization is slight, both on the inside and the outside. They lie at the side of, and slightly behind, the oral cone (Text-fig. 3, *th.app.1*). On their inner surface posteriorly they bear a row of strong comb-like setae, which scrape against the outer wall of the labrum (fig. 7, s., Pl. 35). These are the appendages termed antennules by Yosii in the female of *B. japonicus*, but it seems more likely that they have the identity given above.

#### (iv) The Thoracic Appendages.

There are three pairs of thoracic appendages attached to the first three free thoracic segments behind the head region. The fourth free thoracic segment bears no appendages, so that this species represents a more advanced stage of reduction than *B. maldivensis*, which has a vestigial fourth pair.

Yosii considers that in *B. japonicus* the first two thoracic segments are fused with the head, so that the free thoracic segments are the third, fourth, fifth, and sixth. This explanation seems applicable both to *B. maldivensis* and to this new species.

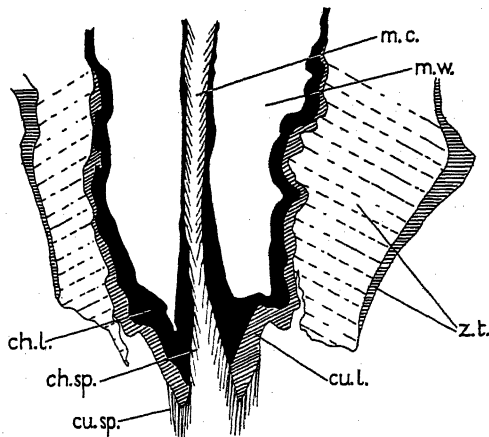
### 3. THE INTERNAL ANATOMY.

#### (a) The Chitinization of the Body-wall.

Both the segmented body and the mantle coils are enclosed in a chitinous wall. The chitin of the mantle coils is of a moderate, uniform thickness (fig. 18, Pl. 37; fig. 20, Pl. 38), but the wall

of the segmented body is unevenly thickened (Text-fig. 1, *a-c*.; fig. 14, Pl. 37). Laterally the chitinous ridge is conspicuous, but elsewhere the layer of chitin is very thin and delicate.

The exoskeleton of Crustacea is made up of an inner chitinous layer and an outer cuticular layer (Yonge, 9). In sections stained with acid fuchsin and Mallory's triple stain the two layers can be distinguished readily, for the chitin stains a bright



TEXT-FIG. 4.

Mantle-tip, at point of attachment of parasite to host. ( $\times 370$ )  
*ch.l.*, chitinous layer; *ch.sp.*, chitinous spines; *cu.l.*, cuticular layer; *cu.sp.*, cuticular spines; *m.c.*, mantle-cavity; *m.w.*, mantle-wall; *z.t.*, zoanthid tissue.

blue in the aniline blue and the cuticle a bright red in the acid fuchsin.

As it is probable that the outer cuticular layer is the most important factor in controlling the passage of substances through the exoskeleton as a whole, it is important to know in the case of a parasite wholly buried within the tissues of its host to what extent this cuticle is present. Serial sections stained in Mallory's stain show that the cuticle is practically absent in *Baccalaureus*. The characteristic red stain is seen only on the mantle tip where the parasite is attached to its host (Text-fig. 4), on the tips of the maxillae, on the caudal fork, and in

traces on the anterior face of the labrum. Elsewhere only the chitinous layer is present. This limited occurrence of cuticle may be important in the absorption of food (see § 3 (f) below).

As recorded by Yosii (10) the chitin of the mantle folds is raised into numerous small papillae. These are scattered all over the mantle coils and penetrate some distance into the enclosing Zoanthid tissue (fig. 8, Pl. 36). Those on the outer coils are not pierced by ducts, but those on the inner coils show fine ducts running through them at their tip (fig. 8, Pl. 36). These ducts are very minute even under the highest magnifications, and it is not possible to determine whether they are actually open at both ends or not; but a careful examination indicates that the former is probably the case. As Yosii states, there is no evidence that they function in nutrition.

Traversing the chitinous ridge and the chitinous and cuticular layers of the mantle tip are ducts of a different form. They run from the body-cavity and open on the outer surface. The inner part of the duct is broad, then there is a spherical dilation followed by a very fine duct leading to the exterior (fig. 9, Pl. 36). The latter may be straight, as in those of the chitinous ridge, or winding, as in the cuticle of the mantle tip. The appearance of these ducts suggests that they might be the ducts of tegumentary glands (Yonge, 9), but an examination of the underlying epidermis does not show any sign of such glands.

#### (b) The Fusion of the Body-wall and Mantle.

The segmented body is fused with the mantle both dorsally and laterally (Text-fig. 1, *d.*). Laterally, it is fused in the region of the adductor muscle, i.e. towards the hinder end of the head-complex. The area of fusion extends back almost as far as the first free thoracic segment, and takes up practically the whole of the lateral wall of the segmented body in this region. It is here that the lateral branches of the gut and the ovaries pass out into the lateral coils.

The dorsal fusion is in the anterior part of the head, slightly anterior to the lateral fusion—though the two areas of fusion overlap to some extent and thus the head region is fused both laterally and dorsally for some distance. The area of dorsal

fusion extends forwards from a point just in front of the origin of the antennules to the most anterior point of the head.

This triple fusion of the anterior part of the head makes investigation of this region impossible except by means of sections of the whole animal. The retractor muscles of the mouth parts and oral cone and the dorso-lateral muscles of the pharynx also come to be attached to the mantle-wall instead of to the dorsal part of the head (cf. § 2 (b) above and § 3 (e) below).

### (c) The Body-cavity.

Immediately below the chitinous layer, in all parts of the segmented body and the mantle coils, is the epidermis. In the segmented body and in the mantle coils of the older specimens this is irregular and consists of flattened cells scattered in a loose connective tissue (fig. 14, Pl. 37; fig. 20, Pl. 38), but in the mantle coils of the younger specimens the epidermis is much better defined (fig. 19, *o.ep.*, *i.ep.*, Pl. 37), and the cells are scattered more evenly, both under the outer and inner chitinous layers.

The body-cavity is filled with a loose connective tissue with scattered cells (fig. 14, Pl. 37). Here and there, particularly round the hind-gut, in the thorax, and round the gut in the distal mantle coils (fig. 20, Pl. 38), are large amoebocytes. These are usually rounded or elliptical in form. They stain a bright red in acid fuchsin, and in many cases are filled with yellowish granules.

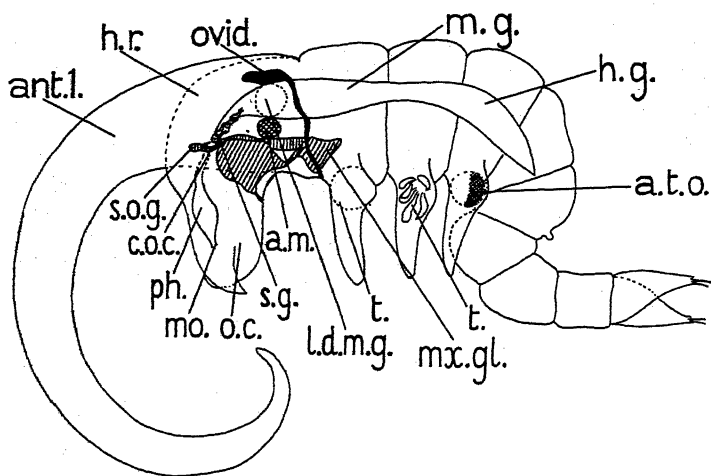
The body-cavity is presumably haemocoelic. Lacaze-Duthiers (6), in his description of *Laura gerardiae*, has described a well-developed vascular system, both in the segmented body and in the mantle, but no trace of this can be found in *Baccalaureus*.

### (d) The Adductor Muscle.

The adductor or digastric muscle occurs in all the members of the Lauridae. In the present species it consists of a chitinous stem running transversely across the segmented body immediately below the gut in the posterior region of the head,



and cone-shaped masses of striated muscle attached to the two ends of the stem and inserted on the lateral mantle-wall (Text-fig. 6, *a.m.*, and fig. 12, Pl. 36). Contraction of these muscles would pull the mantle lobes together, and so move the tips of the mantle, buried in the Zoanthid tissue, from side to side.



TEXT-FIG. 5.

The segmented body, showing the position of the internal organs. ( $\times 60$ ). *a.m.*, adductor muscle; *ant.l.*, antennule; *a.t.o.*, area of opening of testes; *c.o.c.*, circum-oesophageal commissure; *h.g.*, hind-gut; *h.r.*, head region; *l.d.m.g.*, lateral diverticulum of mid-gut; *m.g.*, mid-gut; *mo.*, mouth; *mx.gl.*, maxillary gland; *o.c.*, oral cone; *ovid.*, oviduct; *ph.*, pharynx; *s.g.*, gastric gland; *s.o.g.*, supra-oesophageal ganglia; *t.*, testis.

#### (e) The Gut.

A diagrammatic reconstruction of the principal internal organs is given in Text-fig. 5, and a dorsal view of the same organs in Text-fig. 6.

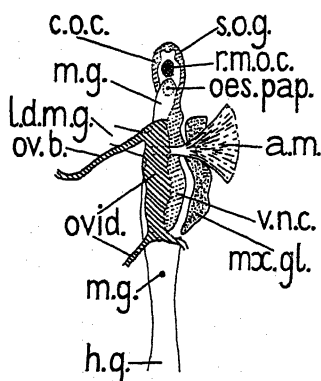
As in the other members of the Lauridae, the gut may be divided, for ease of description, into a central part running within the segmented body, and lateral branches, running out into the mantle coils.

A brief outline of the course and histology of the gut in

*B. maldivensis* has been given in a previous paper (Pyefinch, 8), and Yosii has described it in outline for *B. japonicus*.

The mouth leads directly into a sucking pharynx, contained entirely within the oral cone. The pharynx has three groups of muscles:

- (i) The Lateral Muscles.—These radiate out from the lateral wall and are attached to the lateral walls of the oral cone (fig. 10, *l.m.*, Pl. 36).
- (ii) The Dorso-lateral Muscles.—These run dorsally



TEXT-FIG. 6.

Dorsal view of internal organs. ( $\times 75$ .) *a.m.*, adductor muscle; *c.o.c.*, circum-oesophageal commissure; *h.g.*, hind-gut; *l.d.m.g.*, lateral diverticulum mid-gut; *m.g.*, mid-gut; *mx.gl.*, maxillary gland; *oes.pap.*, oesophageal papilla; *ov.b.*, ovarian bridge; *ovid.*, oviduct; *r.m.o.c.*, retractor muscle of oral cone; *s.o.g.*, supra-oesophageal ganglia; *v.n.c.*, ventral nerve-cord.

and laterally and are attached to the dorsal mantle wall (fig. 10, *d.l.m.*, Pl. 36).

- (iii) The Transverse Muscles, running from one side of the pharynx to the other and wholly contained within its wall (fig. 3, Pl. 35).

Thus the pharynx can be dilated by the lateral and dorso-lateral muscles, and contracted by the transverse muscles.

The narrow oesophagus leads upwards and backwards from the pharynx (fig. 4, Pl. 35) to the digestive portion of the gut.

The opening of the oesophagus into the latter is very small and is situated on the tip of a small papilla.

The gut may be divided into fore-, mid-, and hind-gut. The pharynx and oesophagus, which are lined with a delicate layer of chitin, make up the fore-gut, the anterior part of the central gut and the first part of the lateral gut the mid-gut, and the posterior part of the central gut the hind-gut.

In *B. japonicus* Yosii has divided the gut into oesophagus, stomach, and intestine. He does not support this with any histological evidence, and thus it seemed better, in the present instance, to keep to the more usual terms of fore-, mid-, and hind-gut.

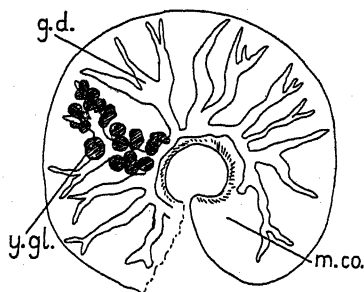
The ducts of the paired gastric glands open into each side of the mid-gut just posterior to the median oesophageal papilla. These glands are bulky and simple, situated in the anterior, upper part of the maxillae, that is, the posterior wall of the oral cone. They are composed of unbranched elements, coiled ventrally but straighter more dorsally (fig. 16, *s.g.*, Pl. 37). The ducts of each gland run upwards and fuse to form a common duct which leads to the mid-gut. The cells stain deeply in aniline blue, cell-walls and nuclei were practically impossible to distinguish, and in places the lumen was difficult to make out. The general appearance of every gland examined suggested considerable secretory activity.

The wall of the mid-gut consists of a deep, columnar epithelium with prominent nuclei (fig. 11, Pl. 36). The upper part of many of the cells was distorted and expanded by large, clear vacuoles (fig. 11, Pl. 36, and fig. 18, *v.*, Pl. 37). In some cases these seem to have burst and so liberated their contents, but in others, particularly where digestion was most active, the whole of the distal part of the cell seemed to be shed, and a vacuolated mass could be seen in the lumen of the gut (fig. 29, Pl. 39, shows an early stage of this). In some specimens also, the cells of the mid-gut were filled with yellowish granules (fig. 31, Pl. 39). At intervals are large goblet cells with dense, granular contents and prominent nuclei (fig. 11, *g.c.*, Pl. 36).

The mid-gut is continued into the lateral coils for a short distance and extends back along the body to the second or

third free thoracic segment. The cells become more irregular, and the lumen becomes much reduced (fig. 14, *m.g.*, Pl. 37).

In horizontal longitudinal sections of the segmented body the boundary between mid- and hind-gut can be distinguished easily. The hind-gut is made up of cubical epithelial cells, with dense contents (fig. 17, *h.g.*, Pl. 37). In the fourth thoracic segment the gut rapidly decreases in diameter, and finally tapers away at the posterior end of this segment. Just before



TEXT-FIG. 7.

Outermost coil of mantle, after clearing in cedar wood oil. ( $\times 25$ .)

*g.d.*, gut diverticulum; *m.co.*, mantle-coil; *y.gl.*, yolk-gland.

this the lumen is crossed by numerous fine strands. The present species agrees with *B. maldivensis* in having no anus, but in both the male and female of *B. japonicus* Yosii has described an anus on the dorsal surface of the fourth abdominal segment. This position seems anomalous.

The lateral branches of the gut arise from the central gut in the region of the adductor muscle. They lead out left and right and then turn sharply upwards (supposing the animal to be so orientated that the dorsal surface of the segmented body is upwards) into the lateral coils. They run round within the outer wall of these, keeping to the inside edge. At intervals branches are given off which traverse the tissue of the mantle (Text-fig. 7).

The distal lateral branches differ considerably in their histology from the branches nearer the segmented body. The epithelium has broken down, and the nuclei, sometimes irregular in outline, lie scattered in a homogeneous mass of protoplasm. In some specimens this part of the gut is loaded with yellowish

granules, and in every case it was surrounded by large amoebocytes (fig. 20, Pl. 38), so that this region may be absorptive.

The differentiation and histology of the gut varies considerably in different specimens. These differences cannot always be correlated with the stage of development as judged by the ovaries and embryos, but in some cases there is some correlation. The description of the gut given above is that of a specimen in which the ovaries were just developing and the mantle coils empty. Fig. 15, Pl. 37, shows a transverse section of the gut, drawn to the same magnification and in the same region as fig. 12, Pl. 36, of a specimen with developing embryos in the mantle folds. The mid-gut of the former specimen resembles the lateral gut of the latter. There is no epithelium, the scattered nuclei are irregular in outline, and the whole gut is reduced in diameter. Although in this case the gut seems so degenerate histologically, in extent it is normal.

In all the specimens described no material of any kind could be found within the gut. In Laura Lacaze-Duthiers found the gut, both in the mantle and in the segmented body, to be full of yellowish granules. Because of this he regarded the gut as being excretory in function. Though yellowish granules were found in the gut in the mantle, and in some cases in the mid-gut of the segmented body (fig. 31, *m.g.*, Pl. 39) these granules were never free in the lumen. There is no other evidence that suggests that the gut might be excretory in *Baccalaureus*; and, in addition, a well-developed maxillary gland is present (§ 3 (*g*), below).

#### (f) The Nature and Source of the Food.

Though the parasite is entirely enclosed within a layer of Zoanthid tissue and the mantle cavity is only in direct contact with the host tissue at its point of attachment, yet the well-developed mid-gut and the active salivary glands argue that digestion of some sort takes place. The chief problems are the nature and source of the food.

The food may be:

- (i) Pieces of Zoanthid tissue, detached by the rocking action of the mantle tips, and taken in through the mouth.

- (ii) Food, previously digested by the Zoanthid, taken in through the mouth.
- (iii) Absorption of food material all over the surface of the parasite.

These problems can be solved only by an investigation of living material, but from a study of preserved specimens these possibilities may be discussed.

The structure of the mouth parts supports the first possibility to some extent. The comb-like setae on the first thoracic appendage and labrum seem adapted for holding pieces of food, and the upturned tips of the maxillae would serve to press such pieces against the mouth. The sucking action of the pharynx would then draw them in. But if pieces of tissue are taken up in this way, it is reasonable to suppose that the mouth parts would sometimes have such pieces adhering to their setae, and that some recognizable contents should be found in the gut; but neither has been found in any of the specimens examined. Again, if large particles were drawn in, they could not pass through the small oesophageal-mid-gut opening.

It is conceivable, however, that if pieces of Zoanthid tissue pass up the mantle cavity, external digestion takes place. If this process were practically complete, then it would be possible for the products of digestion to pass the oesophageal opening, and there would be less likelihood of any contents being found in the gut.

If food materials already digested by the Zoanthid are used and taken in through the mouth, then the method of transport there is obscure, and also it is not easy to see why the mouth parts should be so well developed, unless they are relics of an earlier stage in the life-history which dealt with larger food masses.

The same difficulty occurs in considering the third possibility, the absorption of food all over the surface of the mantle. But there is other evidence which shows that this latter process may take place. As has been pointed out above, there is no cuticular layer in the exoskeleton of the mantle coils, and thus, provided that some external digestion can take place over the mantle surface, there is no barrier to the diffusion of digested material

inwards. If the Zoanthid tissue is carefully examined it is seen that the part in contact with the parasite differs from that farther away. The outer part typically consists of a columnar epithelium, staining deeply in aniline blue and with scattered nuclei (fig. 19, *o.z.t.*, Pl. 37). The inner part consists of irregular masses, sometimes made up of numerous small rounded cells, sometimes more homogeneous, pressed together against the mantle-wall. The thickness of this inner part varies, it is practically absent at the tips of the papillae (§ 3 (*a*), above) but accumulates in the bays between them. The boundary between these two types of tissue is usually distinct.

The outer mantle-wall also shows aggregations of yellowish brown granules, which lie scattered in the inner and the outer epidermis, and in the connective tissue between. The nature of these granules is obscure (fig. 19, *gr.*, Pl. 37).

It thus appears that some change is going on at the surface of the mantle, a change that might possibly have some connexion with the digestion of the Zoanthid tissue at that point.

#### (*g*) The Maxillary Gland.

All the specimens examined showed a well-developed paired maxillary gland (Text-figs. 5, 6, *mx.gl.*). Each gland may be divided into end-sac, communicating duct, and maxillary sac.

The end-sac is large, triangular in transverse section, and situated at the side of and below the ventral nerve-cord, with the base of the triangle directed ventrally (fig. 21, Pl. 38). It extends from the posterior part of the head complex into the first free thoracic segment, and is surrounded by a muscular sheath (fig. 21, *m.s.*, Pl. 38). Its microscopic structure agrees closely with that described by Cannon for *Estheria* and for *Ostracods*. The inner wall is irregular, the nuclei are scattered, and cell-walls can only be distinguished at intervals. The cytoplasm contains many vacuoles, which accumulate until expelled into the lumen, and numerous deeply staining granules.

The end-sac tapers anteriorly, and the communicating duct leads out from its upper anterior end to run obliquely forwards and downwards into the maxillary sac (Text-fig. 5). The histo-

logy of this duct differs considerably from that of the maxillary or end-sac. The boundary between it and the end-sac is very distinct, since the most posterior cells of the duct project backwards into the lumen of the end-sac. These cells were carefully examined, but no trace of a sphincter muscle could be seen. Thus *Baccalaureus* parallels the condition described for the Ostracod *Limnadia* by Nowikoff (7). The rest of the wall of the communicating duct is formed of a cubical epithelium, with moderately prominent nuclei.

The communicating duct is short and leads into the maxillary sac (Text-fig. 5). The latter is thin-walled and runs forwards and downwards. It varies considerably in width, about the middle of its length it extends to the middle line, touching the sac of the opposite side below the nerve-cord (fig. 12, Pl. 36; fig. 23, *mx.s.*, Pl. 38).

About half-way down the maxilla, the maxillary sac narrows considerably and opens by means of a short duct on the posterior lateral edge of the maxilla (figs. 25, 26, Pl. 38).

Parts of this gland have been described in other Ascothoracica. Fowler, in his description of *Petrarca bathyactidis*, says, 'Starting in the oral cone, apparently in a loose network of connective tissue, a duct with a clearly defined lumen runs backwards to open at the base of the (first or) second appendage' (3, p. 112).

It seems that Fowler had seen the posterior part of the maxillary gland. The end-sacs, about the middle of their length, do become large enough to fill the ventral and lateral parts of the body-cavity and so come into contact with the ventral body-wall. Careful examination of serial sections of a number of specimens shows, however, that there is no communication between the end-sacs and the exterior other than by the maxillary sacs and their ducts.

Yosii, describing the female of *B. japonicus*, gives rather a confusing account of 'coelomic cavities'—which divide up into a ventral and two small dorsal branches, the posterior dorsal branch being 'connected with the organ which is said to be the excretory organ'. His diagram ((10.) fig. 5, Pl. 9) shows that he is referring to the maxillary sac.



In the present species this gland is well developed, and shows no signs of degeneration. At the same time it is not easy to see what happens to the excretory products after they have been expelled as the mantle cavity is nowhere in communication with the exterior.

Also, embedded in the connective tissue of the body-cavity, particularly round the gut and the end-sac of the maxillary gland, are dense, homogeneous masses which stain deeply in aniline blue (figs. 21 and 24, *ex.gr.*, Pl. 38). These may be insoluble deposits which are excretory in nature.

#### (h) The Nervous System.

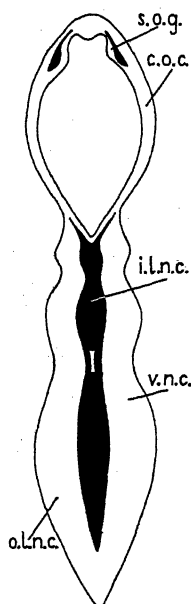
The nervous system consists of small supra-oesophageal ganglia, a pair of circum-oesophageal commissures (fig. 4, Pl. 35; fig. 10, *c.o.c.*, Pl. 36), and a ventral nerve-cord (fig. 12, *v.n.c.*, Pl. 36). Text-fig. 8 shows a reconstruction of this. The ventral cord runs back immediately below the adductor muscle and between the maxillary glands, so that it is always some distance from the ventral surface of the body. It extends as far as the first free thoracic segment.

The general description given above agrees with that of Yosii for *B. japonicus*, except that he terms the ventral nerve-cord 'abdominal ganglion'. As it extends backwards as far as that of the present species there seems no justification for this term.

In transverse section the ventral nerve-cord is seen to consist of two parts. There is an inner core of a mass of tangled fibrils, and an outer sheath of loose-connective tissue, with scattered cells staining deeply in aniline blue (fig. 12, *o.l.n.c.* and *i.l.n.c.*, Pl. 36). Thus the nervous system at this point agrees with that of other Crustacea. The central fibrillar part is not, however, found throughout the system, the shaded part of Text-fig. 8 indicating its extent. Its absence from the posterior tip of the ventral nerve-cord and the circum-oesophageal commissures may be a sign of the degeneration of these parts.

The ventral nerve-cord shows a series of three lateral swellings, of which the posterior is the largest. These may represent ganglia, but as they cannot be correlated with any differences

in internal structure, this seems improbable. There are nerves passing out from the nerve-cord to the adductor muscle. These pass out from the fibrillar core just posterior to the stem of the adductor, and run upwards and outwards to its branches.



TEXT-FIG. 8.

The nervous system, from a reconstruction. ( $\times 310$ .) *c.o.c.*, circum-oesophageal commissure; *i.l.n.c.*, inner fibrous layer; *o.l.n.c.*, outer layer of nerve-cord; *s.o.g.*, supra-oesophageal ganglion; *v.n.c.*, ventral nerve-cord.

### (2) The Reproductive Organs.

All the specimens were hermaphrodite. Yosii describes *B. japonicus* as being dioecious. This explanation seems to be based on a mistaken interpretation of his material, but the point will be discussed in greater detail below.

A series of seven specimens, selected at random, were specially investigated in an attempt to trace the general course of growth and the stages of development of the gonads in the adult. The results are given in Table 2 below:

TABLE II.

<i>Specimen No.</i>	<i>Position in Polyp.</i>	<i>Size in cm.</i>	<i>Development of Yolk-glands.</i>	<i>Development of Ova.</i>	<i>Development of Testes.</i>	<i>Contents of Mantle.</i>
*17	Top	0.61	Full	Ova in mantle folds	Rosette stage	None
20	Top	0.58	Becoming depleted	—	Testes depleted	Nauplii and late development stages
24	Top	0.53	Full	Ova in mantle folds	—	None
10	Top	0.50	Outer coil empty	—	Free sperm	Nauplii
*15	Top	0.44	Patchily developed	Ova in mantle folds	Rosette stage	None
5	Top	0.42	Full	Budding from ovarian bridge	Free sperm	Developing ova, early stage
7	Top	0.40	Full	In mantle folds, on ovarian bridge with yolk	Rosette stage	None

\* Specimens 15 and 17 were found in the same polyp.

It seemed possible that the size of the animal could be taken as a rough indication of its stage of development, and in the description of *B. maldivensis* (8) size was taken as a rough criterion of age. The specimens analysed in Table 2 offer no support for this view, as even specimens differing widely in size, e.g. spp. 7 and 17, had reached the same stage of development.

Table 2 also shows that free spermatozoa are produced late on in the developmental history of the ova, e.g. spp. 10 and 15. This means that the ova are probably not fertilized until they are just ready to pass out into the mantle cavity, that is, that fertilization must take place either in the oviduct or in the mantle cavity itself.

Specimens 15 and 17 were found in the same polyp, and the Table shows that they are practically at the same stage of development, and thus the aggregation of two or more parasites in the same polyp is purely a chance result, rather than the development of offspring side by side with the parent.

The reproductive organs will be considered under three headings:

- (i) The yolk-glands.
- (ii) The ovaries.
- (iii) The testes.

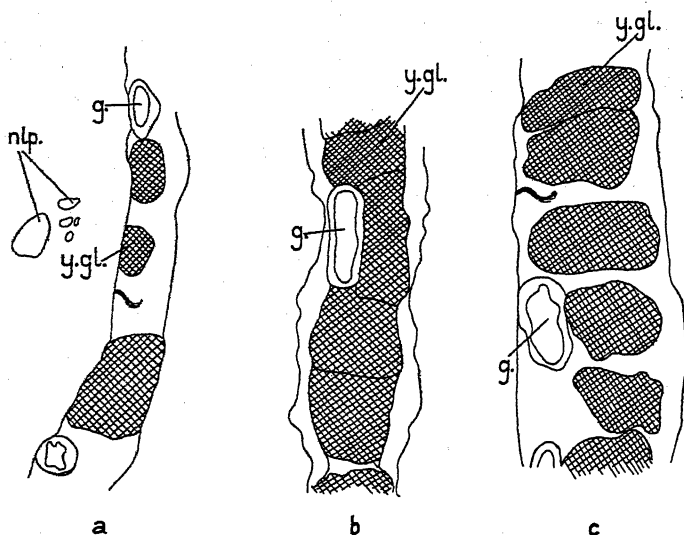
(i) The Yolk-glands.

In a previous paper (Pyefinch, 8) these were described as ovaries, but in the present material it has been possible to distinguish between ovaries and yolk-glands. The latter are found all over the outer mantle wall, lying between the gut and the outer chitinous layer (fig. 32, Pl. 39). In surface view they appear as rounded nodules lying over the branches of the gut (Text-fig. 7, *y.gl.*). Usually they lie entirely lateral to the gut, but when fully developed they may extend round three sides (fig. 32, Pl. 39) of the latter.

In specimens which have no developing embryos in the mantle cavity the yolk-glands are full and expanded (Text-fig. 9, *c*), where early developmental stages are present in the lumen of the mantle folds the glands are moderately full (Text-fig. 9, *b*),

and when nauplii are present they may be much depleted (Text-fig. 9, *a*). Thus there is a positive correlation between the development of the yolk-glands and the period of maximum egg production.

In section the yolk-glands consist of masses of globules, both small and large, staining deeply in acid fuchsin (fig. 28, Pl. 39).



TEXT-FIG. 9.

Sections of the mantle-wall, showing development of yolk glands. (For description see text.) ( $\times 130$ .) *g.*, gut; *nlp.*, nauplius; *y.gl.*, yolk-gland.

In many glands there is a peripheral ring of lacunae, but no trace of a duct.

## (ii) The Ovaries.

These consist of two parts, the larger part lying in the mantle coils, as a branching structure roughly following the course of the hind-gut and its branches. These branched structures pass into the segmented body in the region of the adductor muscle, where they are connected across the middle line by the narrow bridge-like second part (Text-fig. 6, *ov.b.*). The morphology of

the ovaries is thus similar to that of *Laura*, *B. japonicus*, and *Petrarca*.

In a mature specimen a large median sinus-like oviduct runs backwards from the ovarian bridge to the posterior edge of the head complex, where it gives off, right and left, narrow oviducts, which run downwards and backwards, opening on the ventral surface of the segmented body, just in front of the second thoracic appendage (fig. 31, Pl. 39). This oviduct is distinct only in specimens containing fully developed eggs.

The eggs are budded off principally from the germinal epithelium lining the ovarian bridge. Budding may take place elsewhere, but it has not been observed. The fully developed ovum is a large, round cell, with a large nucleus, containing a deeply staining nucleolus, and with vacuolated cytoplasm (fig. 27, *ov.*, Pl. 39).

In this stage the ova pass out into the mantle coils where they lie between the hind-gut and the yolk-glands, so that they are pressed against the inner wall of these glands (fig. 28, Pl. 39). Whilst in the mantle the ovum is covered with yolk, and then passes back to the ovarian bridge (fig. 29, Pl. 39). From here the ovum passes down the median oviduct, and out through one of the lateral oviducts, causing considerable distortion of the latter on its way (fig. 30, Pl. 39).

The ova are probably fertilized when in the oviduct or in the lumen of the mantle coils, and in one specimen examined a bundle of sperm was seen in the base of the oviduct.

### (iii) The Testes.

Every specimen examined showed testes in some stage of development, as well as ovaries. The testes are situated at the proximal end of the appendages of the first three free thoracic segments, and in mounts of the whole body appear as rounded dilatations (Text-fig. 5, *t.*).

The testes open by a number of pores, scattered over the outer and posterior part of the testicular region of the appendage (Text-fig. 5, *a.t.o.*) so that a surface mount of this part appears grid-like. Immediately below each opening is a small chamber, lined by chitin, into which the sperm pass before they are

expelled (fig. 35, *cav.*, Pl. 40). The tubules of the testis do not communicate directly with these chambers, but several tubules open into a long, narrow chitin-lined duct, which may be described as a vas deferens (fig. 37, *v.d.*, Pl. 40). The wall of the vas deferens is formed of a narrow band of tissue with a large number of small nuclei, and so is distinct from the tubules themselves (fig. 37, *v.d.* and *t.t.*, Pl. 40).

The tubules of the testis, which twist in and out but do not branch, are suspended in a loose, vacuolated connective tissue (fig. 33, Pl. 40). The course of development of the spermatozoa has been made out only in the broadest outline. The earliest recognizable stage is the spherical spermatogonium which is successively uni-, bi-, and multi-nucleate (fig. 33, *spg.*, Pl. 40). All the nuclei of the multinucleate stage except one migrate to the periphery of the cell, which is now conspicuously large. Division takes place with the formation of several small cells surrounding a protoplasmic mass. This is the rosette stage (figs. 33 and 34, *r.s.*, Pl. 40). In the early stages of this, the outer cells are multinucleate, later they become uninucleate. It has not been possible to find with any certainty any stage between this late rosette stage and the fully developed filiform spermatozoa, but fig. 36, Pl. 40, and fig. 34, Pl. 40, may represent intermediate stages, the former showing a developing rosette, and the latter a still later stage, in which the spermatozoa are forming.

The spermatozoa resemble those of other Cirripedes in being long and filiform. They are formed in bundles (fig. 35, Pl. 40), and pass out arranged in this way. In this *Baccalaureus* differs from *Laura*, as in the latter the spermatozoa pass out serially through the minute pores of the testes.

In *B. japonicus* a 'penis-like projection' has been described on the ventral surface of the first abdominal segment, but there is no trace of this in *B. maldivensis* or the present species.

#### 4. DEVELOPMENT AND LARVAL STAGES.

Three stages of development could be distinguished:

- (i) Ova recently liberated.

(ii) Late developmental stages.

(iii) The Nauplii.

The preservation of the material was not good enough to allow of any detailed investigation of the two earlier stages, so that only their more important characters can be described.

The first stage showed the ovum, granular in appearance embedded within a mass of yolk, and both enclosed in a delicate membrane. Lacaze-Duthiers found in *Laura* that the more mature ova had smaller yolk-granules than the younger forms, but this does not hold for *Baccalaureus*.

The stage placed as second above was much later. The yolk had entirely disappeared, and the appendages of the nauplii could be distinguished through the outer membrane.

Finally, in several specimens nauplii were free in the mantle coils. In all essential details they agree entirely with those of *B. maldivensis*. Table 3 below gives a comparison of some of the principal characters of the nauplii of the Lauridae, so far as they are known. The characters chosen for comparative purposes are:

(i) The presence or absence of frontal spines.

(ii) The number of terminal setae on each ramus of each appendage.

(iii) The character and number of the basal spines.

These characters seem reasonably constant, and at the same time are easily verified. The number and type of the sub-terminal and lateral setae on each appendage also seem to vary from one species to another, but are not so easily verified.

The nauplius of *B. japonicus* differs from those of other members of the Lauridae in having frontal spines. The nauplii of *Laura gerardiae*, *B. japonicus*, and *B. maldivensis* also show considerable differences among themselves, but the Table brings out clearly the close similarity between *B. maldivensis* and the present species. They differ only in the number of terminal setae on the upper ramus of the second appendage, and even this difference is not constant, for some of the present specimens show three terminal setae and some two terminal and one sub-terminal. The implications of this close resemblance are further discussed below.



TABLE 3. A Comparison of Naupliar Characters in the Lauridae.

Genus and Species.	Appendage 1.		Appendage 2.						Appendage 3.						
	Frontal spines.	Terminal setae.	Upper Ramus.		Lower Ramus.				Upper Ramus.		Lower Ramus.		Type of spine.		
			Terminal setae.	Spines.	Terminal setae.	Spines.	Terminal setae.	Spines.	Terminal setae.	Spines.					
Laura gerardiae . . .	—	1	0	1	0	1	6	0	Si	1	0	1	4	2	Si
Baccalaureus japonicus .	+	3	0	2	0	3	4	4	Si+Se	1	0	3	2	2	Se+Si
Baccalaureus maldivensis	—	2	0	3	0	2	0	2	Si	3	0	2	0	2	Si
Baccalaureus hexapus .	—	2	0	2 or 3?	0	2	0	2	Si	3	0	2	0	2	Si

Se = setose; Si = simple.

In *Laura gerardiae* the contents of the mantle showed every stage of development at once, but in the present species, though there were slight individual differences, all the contents of the mantle cavity were approximately at the same stage of development. However, it is possible that several batches of ova may be produced, as specimens with developing ova in the mantle coils also showed ova budding from the ovarian bridge (see Table 2).

No further larval stages were seen, and the method of liberation of the nauplii remains unknown. There is no evidence for their direct liberation by the bursting of the mantle-wall.

### 5. THE SYSTEMATIC POSITION.

The specimens described above differ from *B. maldivensis* in:

- (i) The loss of the maxillulae (first maxillae).
- (ii) The loss of the appendages of the fourth free thoracic segment.

In the present stage of our knowledge of the mutual relationships of the various members of the Ascothoracica, the specimens described above must be placed in a new species of the genus *Baccalaureus*, for which I propose the name *B. hexapus*.

The characters of the genus *Baccalaureus*, revised to include this new species, are thus:

Ascothoracica with a mantle of median and lateral lobes. Four free thoracic segments. Thoracic appendages uniramous and unsegmented. Each ramus of the caudal fork with three stout spines. A chitinous ridge along the side of the thorax.—*Baccalaureus*.

- (i) Lateral lobes of mantle sac-like.

Thoracic appendages sub-equal. Penis-like projection from the first abdominal segment. Anus on the dorsal surface of the fourth abdominal segment.—*B. japonicus*.

- (ii) Lateral lobes of mantle coiled.

No penis-like projection of the first abdominal segment. No anus.

- (a) Appendages of fourth free thoracic segment and maxillulae reduced.—*B. maldivensis*.
- (b) No appendages on fourth free thoracic segment. Maxillulae absent.—*B. hexapus*.

## 6. DISCUSSION.

Yosii has described male and female forms in *B. japonicus*. The female is embedded in the tissues of the Zoanthids *Palythoa* and *Zoanthus*, whereas the male is found as an ectoparasite on the same genera. The existence of males and females is surprising, since *Laura gerardiae* and *B. hexapus* are both hermaphrodite, and a re-examination of the *maldivensis* material indicates that this is probably hermaphrodite also.

In his description Yosii makes certain statements which make it difficult to accept his interpretation. In the female are ovoidal bodies in the appendages of the first three free thoracic segments—'like those found in the legs of *Laura* . . . filled homogeneously with bundles of fibrils. . . . The fibrils resemble the sperm, but more histological study must be done to give a definite determination'. Again, in the male he says '. . . the sperms I have not been able to identify with certainty, but the structures which fill the testes and vas deferens may be of that nature', and 'The inner side of the ventral halves of the shells are filled with large cells with large, round nuclei which resemble young eggs', and thirdly, 'It seems probable that the animal is an intersex and not a pure male'.

To summarize his description, the sex of each type seems to be ill-defined, and each seems to be hermaphrodite rather than unisexual. His diagram of the female ((10.) fig. 4, Pl. 9) closely resembles the segmented body of the other species of *Baccalaureus*, but the diagram of the male ((10.) fig. 8, Pl. 10) closely resembles the typical cypris larva of a Thoracican. The specific points of agreement are the bi-valved 'shell', and the well-developed, segmented antennules which are used for attachment, but it resembles a cypris also both in general bodily form, and the completely segmented thorax, and shows a primitive character in the segmentation of the appendages.

It is suggested, therefore, that Yosii's material can better be interpreted as late cypris larvae and adult stages, rather than as males and females. If this suggestion were substantiated by an investigation of the development of *B. japonicus*, the results would evidently be important because it would then be possible for the first time to give the complete life-history of an Ascothoracican, from the nauplius through the cypris up to the adult, and thus finally to establish the Ascothoracica as members of the Cirripectida.

The question of specific differences within the group has been discussed in a previous paper when it was suggested that differences termed specific might only be due to a difference in the stage of development. The present species supports this suggestion, as the nauplii of *B. maldivensis* and *B. hexapus* are practically identical, and the differences between the adults are differences of reduction. Thus *B. hexapus* may well be only a later stage of *B. maldivensis*.

Finally, a description of the internal anatomy can only indicate many of the most important problems in the biology of these animals. This can only be completed by an investigation of the living animal, which would shed light on the method of feeding, the source of the food, the destination of the excretory products, the mode of liberation of the nauplii, and other physiological problems of importance.

In conclusion, I must add my thanks to Dr. L. A. Borradaile for his kindness in reading the manuscript of this paper, and also for his interest and advice during its preparation.

## 7. SUMMARY.

1. An account is given of the external and internal anatomy of a new species of the genus *Baccalaureus*.

2. The animal has a well-developed oral cone, consisting of an anterior labrum, paired mandibles, and paired maxillae. The histology of the gut, both in the segmented body and in the lateral coils, is described in detail.

3. A typical maxillary gland is present.

4. There are supra-oesophageal ganglia, circum-oesophageal commissures, and a short median ventral nerve-cord.

5. All the specimens are hermaphrodite. The ovary consists of a median bridge above the gut and branches in the lateral coils. Yolk-glands also are present. The testes fill the proximal parts of the appendages of the first three free thoracic segments. The spermatozoa are filiform, and are ejected in bundles.

6. Fertilization takes place in the lumen of the mantle coils or in the oviduct. Development as far as the nauplius takes place in the former, but the mode of liberation of these larvae has not been ascertained.

7. The characters of the nauplius are compared with those of other members of the Lauridae.

8. Previous work on the anatomy of the genus is discussed, and a reinterpretation of the results suggested.

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## EXPLANATION OF PLATES 35-40.

## LETTERING.

*ant. 1*, antennule; *ant. 2*, antenna; *am.*, amoebocyte; *a.m.*, adductor muscle; *cav.*, cavity within thoracic appendage; *c.d.*, communicating duct; *ch.l.*, chitinous layer; *ch.p.*, chitinous papilla; *ch.r.*, chitinous ridge; *ch.th.app.*, chitin of thoracic appendage; *c.o.c.*, circum-oesophageal commissure; *c.s.*, comb-like setae; *c.t.*, connective tissue; *d.*, duct through chitinous papilla; *d.l.m.*, dorso-lateral muscles; *d.w.*, dorsal wall of segmented body; *ep.*, epidermis; *e.r.s.*, early rosette stage; *e.s.*, end-sac of maxillary gland; *ex.gr.*, excretory granule(?); *g.*, gut; *g.c.*, goblet cell; *gr.*, granules; *g.w.*, gut-wall; *h.g.*, hind-gut; *i.ch.l.*, inner chitinous layer; *i.ep.*, inner epidermis; *i.l.n.c.*, inner fibrous layer; *i.m.w.*, inner mantle-wall; *i.p.md.*, inner plate of mandible; *i.z.t.*, inner layer of zoanthid tissue; *lbm.*, labrum; *l.d.m.g.*, lateral diverticulum of mid-gut; *l.f.*, lateral fusion of segmented body; *l.m.*, lateral muscle; *l.r.s.*, late rosette stage; *l.w.*, lateral body-wall; *m.b.*, muscle-band; *md.*, mandible; *m.g.*, mid-gut; *m.r.*, mouth region; *m.s.*, muscle-sheath of end-sac; *mx.*, maxilla; *mx.gl.op.*, opening of maxillary gland; *mx.s.*, maxillary sac; *mx.s.d.*, duct of maxillary sac; *ncl.*, nucleolus; *nl.*, nucleus; *o.ep.*, outer epidermis; *oes.*, oesophagus; *o.l.md.*, outer lobe of mandible; *o.l.n.c.*, outer layer of nerve-cord; *ov.*, ovum; *ov.b.*, ovarian bridge; *ovid.*, oviduct; *o.z.t.*, outer layer of zoanthid tissue; *p.*, peritoneal layer; *ph.*, pharynx; *rm.md.*, retractor muscle of mandible; *rm.mx.*, retractor muscle of maxilla; *rm.o.c.*, retractor muscle of oral cone; *rm.th.1*, retractor muscle of first thoracic appendage; *r.s.*, rosette stage; *s.*, setae; *s.g.*, gastric gland; *s.m.*, setae of mantle; *sp.*, spermatozoa; *spg.*, spermatogonia; *st.a.m.*, stem of adductor muscle; *t.c.*, terminal cell of communicating duct; *th.1*, first thoracic appendage; *t.op.*, opening of testis; *t.t.*, tubule of testis; *v.*, vacuole; *v.d.*, vas deferens; *v.m.*, vacuolar mass; *v.n.c.*, ventral nerve-cord; *v.w.*, ventral wall of body; *y.gl.*, yolk-gland; *z.t.*, zoanthid tissue.

## PLATE 35.

- Fig. 1.—Transverse section of left antennule.  
 Fig. 2.—Transverse section of right antennule (from another specimen).  
 Fig. 3.—Vertical section of oral cone, showing mouth region.  
 Fig. 4.—Vertical section of oral cone, showing mouth parts.  
 Fig. 5.—Vertical section of labrum, showing comb-like setae.  
 Fig. 6.—Vertical section of maxilla.  
 Fig. 7.—Vertical section of thoracic appendage and labrum.

## PLATE 36.

- Fig. 8.—Section of outer wall of inner mantle coil.  
 Fig. 9.—Transverse section of chitinous ridge, showing ducts.  
 Fig. 10.—Vertical section of oral cone, in the region of the pharynx.

Fig. 11.—Transverse section of anterior part of mid-gut.

Fig. 12.—Transverse section of mid-gut, at the point of origin of the lateral diverticula.

Fig. 13.—Transverse section of segmented body, showing the position of attachment of the antenna.

#### PLATE 37.

Fig. 14.—Transverse section of thorax.

Fig. 15.—Transverse section of mid-gut, at the point of origin of the lateral diverticula.

Fig. 16.—Vertical section of salivary gland.

Fig. 17.—Transverse section of abdomen, showing hind-gut.

Fig. 18.—Transverse section of mid-gut in mantle coil.

Fig. 19.—Transverse section of surface of mantle coil.

#### PLATE 38.

Fig. 20.—Transverse section of gut in mantle coil.

Fig. 21.—Transverse section of end-sac of maxillary gland.

Fig. 22.—End-sac and communicating duct.

Fig. 23.—Maxillary sac.

Fig. 24.—Transverse section of body-cavity, showing excretory granules.

Fig. 25.—Vertical section of maxilla, showing base of maxillary sac and its duct.

Fig. 26.—Wall of the maxilla, showing opening of maxillary gland.

#### PLATE 39.

Fig. 27.—Transverse section of ovarian bridge.

Fig. 28.—Transverse section of yolk-gland and ova.

Fig. 29.—Transverse section of ovarian bridge, showing ovum and yolk.

Fig. 30.—Transverse section of oviduct, with ovum within.

Fig. 31.—Transverse section of oviduct, empty.

Fig. 32.—Transverse section of mantle coil, showing arrangement of yolk-glands round gut diverticula.

#### PLATE 40.

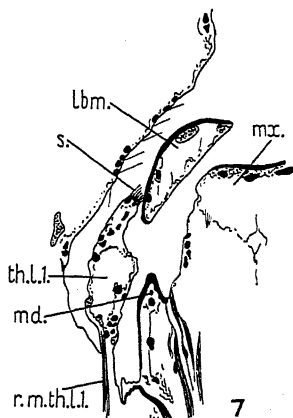
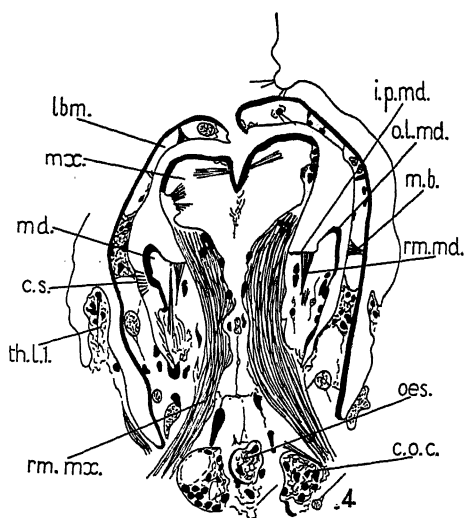
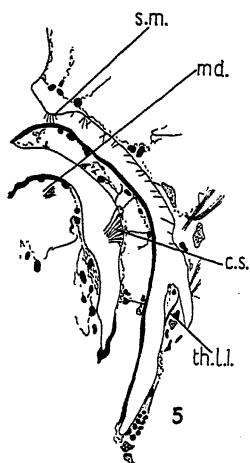
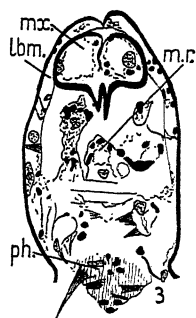
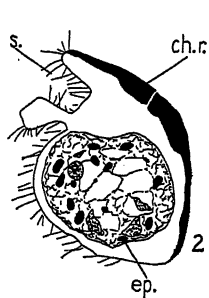
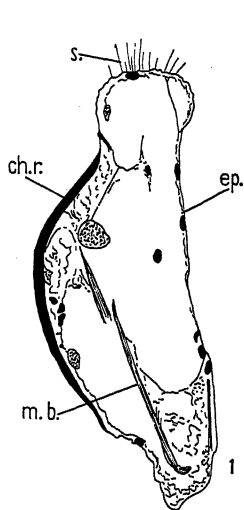
Fig. 33.—Transverse section of thoracic appendage, showing testis tubule.

Fig. 34.—Transverse section of thoracic appendage, showing late stage of testis.

Fig. 35.—Transverse section of testis, with spermatozoa.

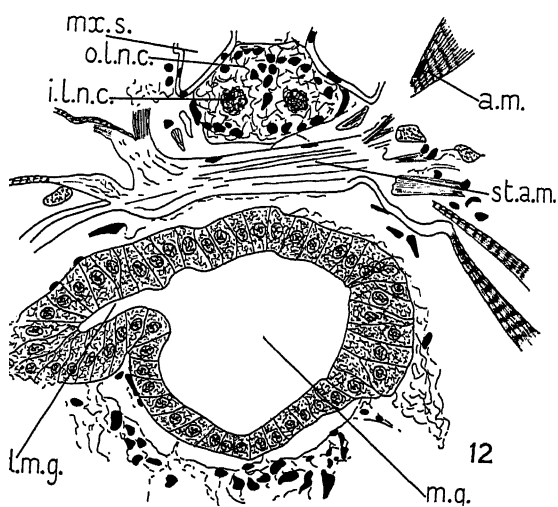
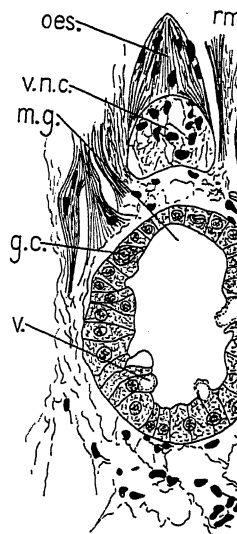
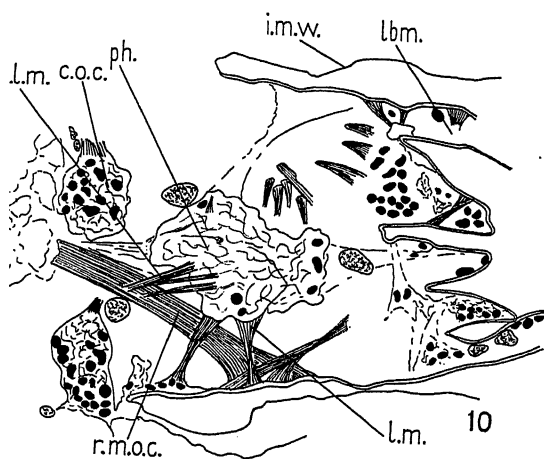
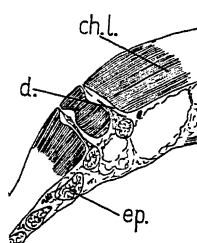
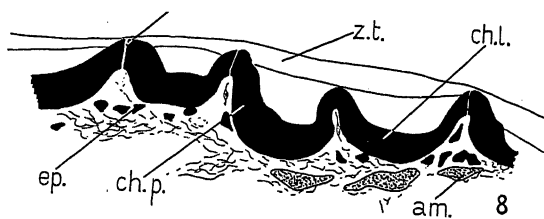
Fig. 36.—Transverse section of testis.

Fig. 37.—Transverse section of testis, showing tubules and vas deferens.

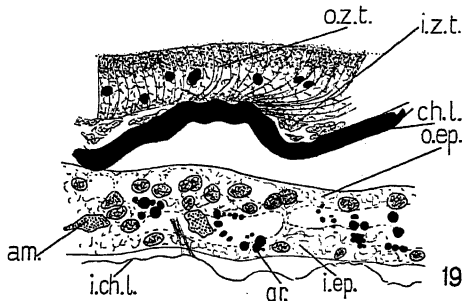
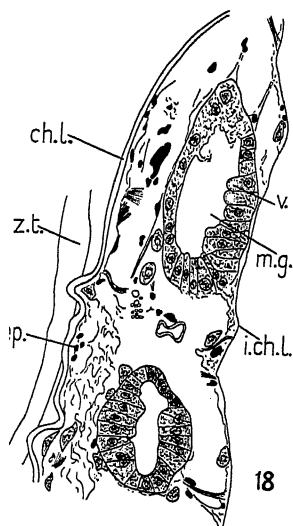
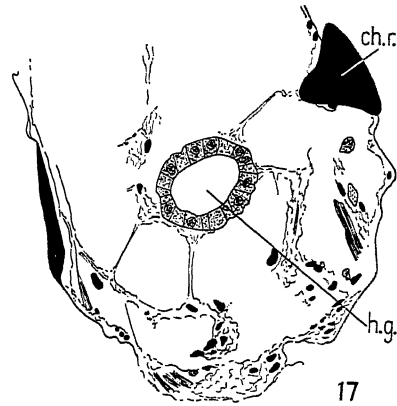
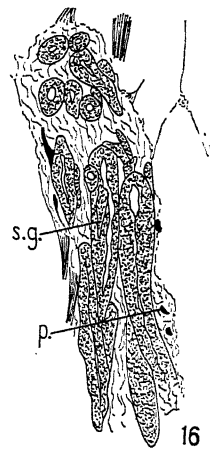
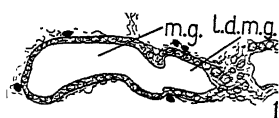
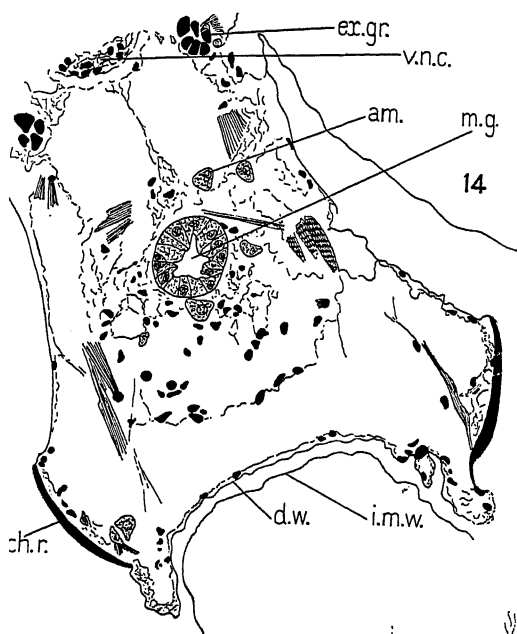




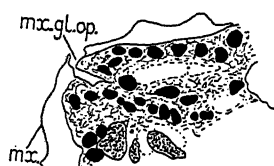
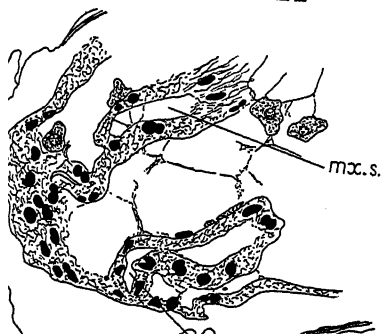
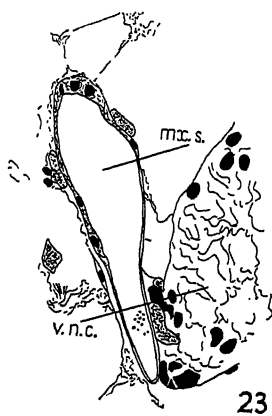
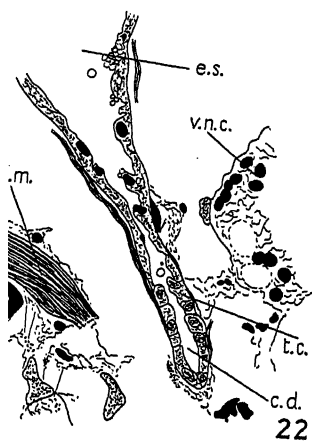
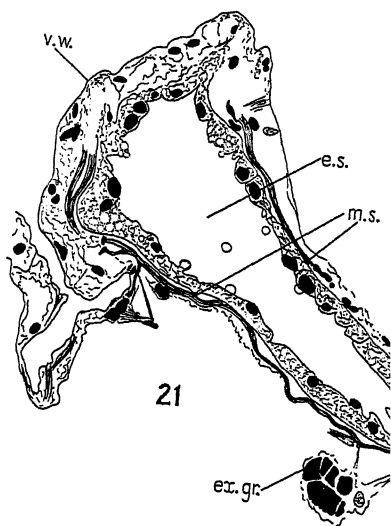
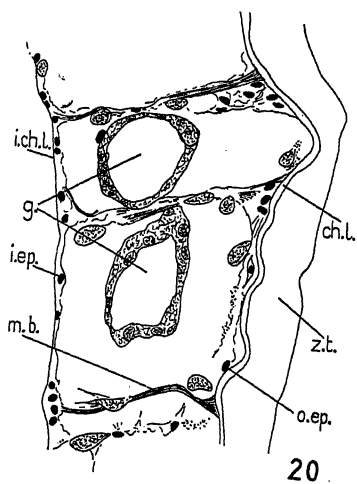




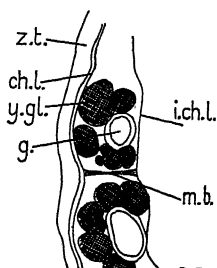
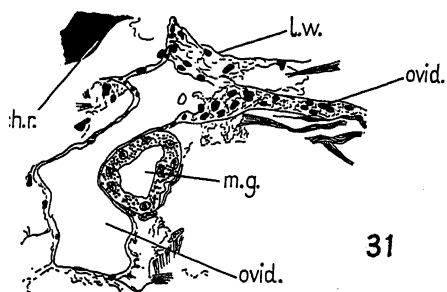
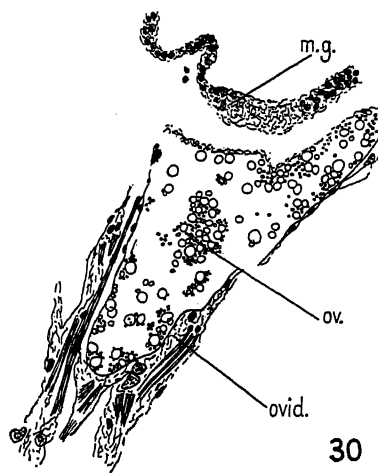
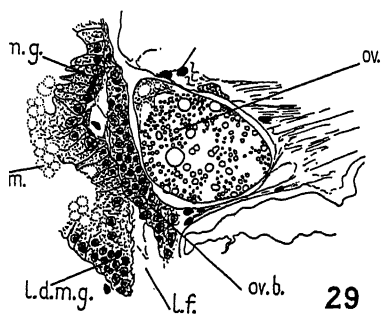
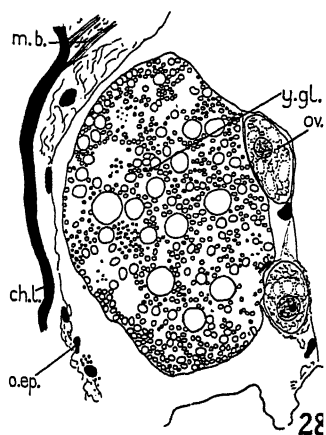
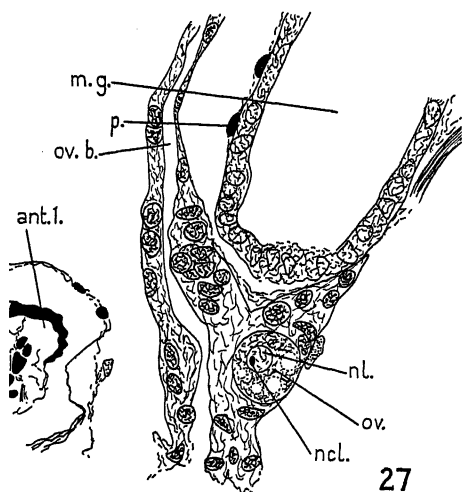






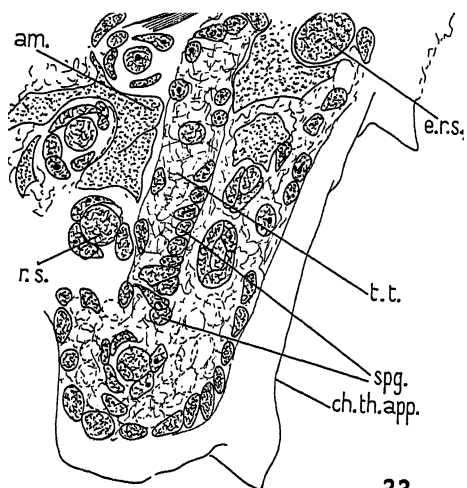




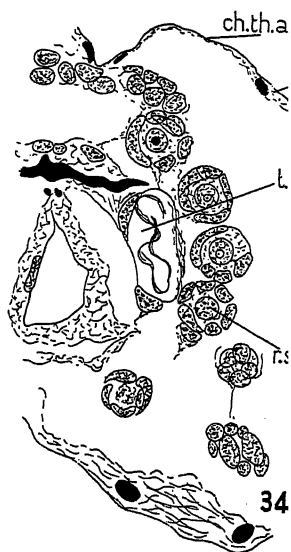




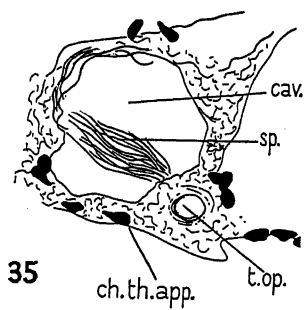




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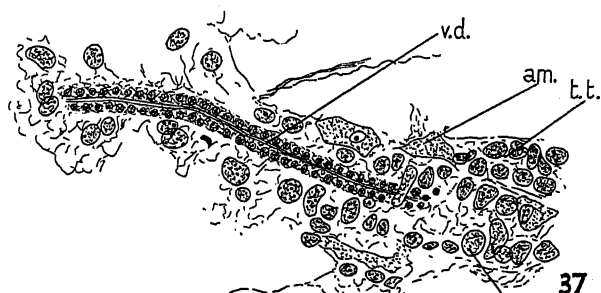
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35



36



37



On the Primitive Streak and associated  
structures in the Marsupial  
*Bettongia cuniculus*.

By

T. Kerr, M.A.,

Assistant in the Zoology Department, Queen's University of Belfast.

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With Plates 41 and 42 and 18 Text-figures.

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MATERIAL AND TECHNIQUE.

In a previous paper (1934) stages were described in the development of Diprotodont Marsupials, particularly *Bettongia cuniculus*, up to the appearance of the primitive streak, and in the present paper the work is continued. As already stated, the material was collected by Professor T. Thomson Flynn from animals taken in the wild in Tasmania during the period 1920 to 1929; it forms part of the Marsupial and Monotreme material obtained by him from grants allowed by the Ralston Trustees and by the Royal Society. I would like to express once again my gratitude to Professor Flynn for the opportunity of examining this most interesting material, and for his very kind advice and help during the course of the work; towards its completion I had the advantage of the invaluable criticism of Professor J. P. Hill, F.R.S., and I am greatly indebted to him both for this and for the generous way in which he extended to me the facilities of his Department in University College, London, during part of the research. This included the photography of the sections, so successfully achieved by Mr. F. J. Pittock from very difficult material.

The part of the material considered here consisted of complete blastocysts, and of series of sections cut in Tasmania by Professor Flynn, as detailed below; the dates given refer to when the blastocysts were obtained from the animals. All the blastocysts except the last were fixed in Bouin's fluid. Sections prepared here, except of the last, were cut at  $5\mu$  and stained

with iron haematoxylin; the shell-membrane in these later stages is much less of a hindrance to section cutting than in the earlier stages. All the complete blastocysts were photographed for reference, and these photographs have been reproduced when they show details of interest. The text-figures of the complete embryonal areas were prepared from graphs made from the series of sections. The method of outlining the area in which mesoderm cells occur has the obvious disadvantage of giving no clue to their concentration, but this can be to some extent discounted by comparisons with the appropriate text-figures and plates.

The measurements given in the following table are, wherever possible, taken from complete blastocysts; it will be noticed from the text-figures that a certain amount of shrinkage has sometimes occurred. Where the blastocysts were not quite spherical maximum and minimum diameters are given.

#### TABLE OF STAGES.

Blastocyst I, 3/9/24. Diameters 1.32 mm. and 0.99 mm.; embryonal area circular, diameter 0.90 mm. Dimensions of proliferative area, 0.06 mm. by 0.06 mm. (approximate maximum length and breadth). Cut transversely.

Blastocyst II, 14/9/24. Diameter 1.40 mm.; embryonal area circular, diameter 1.06 mm. Length of developing primitive streak 0.35 mm. Cut into transverse sections of  $8\mu$  by Professor Flynn.

Blastocyst III, 16/8/28. Diameter 1.60 mm.; embryonal area circular, diameter 1.22 mm. Length of primitive streak 0.65 mm. Cut into longitudinal sections of  $5\mu$  by Professor Flynn.

Blastocyst IV, 18/9/24. Diameter 1.88 mm.; embryonal area circular, diameter 1.52 mm. Length of primitive streak 0.74 mm. Cut transversely.

Blastocyst V, 6/9/24. Diameter 2.07 mm.; embryonal area apparently circular, diameter about 1.58 mm. Length of primitive streak 1.13 mm. Cut longitudinally.

Blastocyst VI, 19/9/24. Diameters 2.23 mm. and 2.13 mm.; embryonal area oval, 1.63 mm. by 1.49 mm. Length of primitive streak 0.96 mm. Cut transversely.

Blastocyst VII, 29/7/25. Very collapsed specimen, diameters 2.76 mm. and 1.97 mm.; embryonal area apparently circular, diameter about 1.50 mm. Length of primitive streak 0.89 mm. Cut longitudinally.

Blastocyst VIII, 21/9/26. Diameter 3.76 mm.; embryonal area oval, 1.97 mm. by 1.62 mm. Length of primitive streak 1.24 mm. Cut transversely.

Blastocyst IX, 11/9/24. Diameter 4.40 mm.; embryonal area slightly pear-shaped, 3.10 mm. by 2.20 mm. Length of primitive streak 1.92 mm. Cut longitudinally.

Blastocyst X, 14/9/24. Uterus fixed entire in Carnoy's fluid and then preserved along with the parts of the blastocyst in absolute alcohol. External diameter of complete blastocyst about 5.5 mm.; embryonal area shaped like body of fiddle, 5.12 mm. in length, 1.72 mm. and 1.28 mm. in maximum and minimum breadth. Length of primitive streak 1.68 mm. Cut transversely.

#### INTRODUCTION.

Few descriptions have been published of the primitive streak in Marsupials. Selenka (1887) figures three blastocysts of *Didelphys virginiana* of about the same age (stated to be about 48 hours, and all about 2 mm. in diameter) showing primitive streak stages, but gives only a very brief description of them. The embryonal area is shown as slightly pear-shaped, the lateral mesoderm well established, and a head-process present. In his figures of sections of these blastocysts the primitive groove is very slightly developed as compared with corresponding stages in *Bettongia*, and the appearance of the primitive streak is different in detail. Selenka's next stage of *Didelphys* is stated to be 64 hours old and has three somites. Later (1892) he published descriptions of stages obtained from females of *Hypsiprymnus* (= *Bettongia*) *cuniculus* which were kept in the animal-house at Erlangen. The earliest is a 2 mm. blastocyst said to be about 2 days old, with a well-formed primitive streak. In it he describes the primitive groove as bifurcating posteriorly, and also as widening in the region of Hensen's node and bifurcating anteriorly;

the surface-view figure in which these bifurcations show so clearly (fig. 1, Pl. xxxii) is to some extent reconstructed from sections, but from one of the sections figured (fig. 10, Pl. xxxii) these anterior grooves show simply as irregular folds, and it is concluded from their absence anteriorly and their rare appearance posteriorly in the blastocysts to be described that they do not have the theoretical significance that Selenka attached to them. His figure through apparently about the middle of the streak (fig. 2, Pl. xxxi) presents the normal appearance. His next stage is a 'three day' old blastocyst with ten somites. Wilson and Hill (1907) describe a late primitive streak stage in *Perameles obesula*, in which the streak itself is 5.8 mm. long, and in which the head-process and prochordal plate are well developed. A gap therefore exists in our knowledge of Marsupial development which is to some extent filled by the material here described.

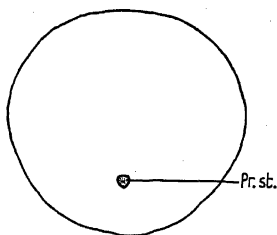
#### DESCRIPTION OF STAGES.

Stage I (diameters 1.82 mm. and 0.99 mm.).

In this stage the ectoderm and endoderm form completely continuous layers round the blastocyst, as indeed they have done from a considerably earlier stage. The ectoderm is throughout much the thicker and denser layer of the two; its differentiation into embryonal and extra-embryonal portions is quite distinct, the former thinning abruptly all round its edge as it passes into the latter (Text-fig. 2). The thickness of the embryonal portion is approximately 0.014 mm. and that of the extra-embryonal approximately 0.003 mm. The shape of the ectoderm nuclei varies, the extra-embryonal being somewhat flattened and the embryonal more or less rounded; the ectoderm throughout is but one cell thick. In the earlier stages the endoderm is a uniform layer and even now there is, except for the annular zone, but little difference between embryonal and extra-embryonal portions; both are greatly attenuated, a small amount of cytoplasm is associated with each nucleus, but between nuclei it becomes drawn out into thread-like processes. These general characteristics of ectoderm and endoderm remain much the

same for later stages, except that the extra-embryonal endoderm tends to become less attenuated.

In this blastocyst has made its appearance a small area of thickened ectoderm, about 0.06 mm. in its greatest length and in its greatest breadth, situated rather nearer to the periphery than to the centre of the circular embryonal area (Text-fig. 1). From its position it is obviously destined to form part of the middle of the primitive streak. Investigations by Hubrecht (1890) on *Sorex*, Assheton (1894) on the Rabbit, and Streeter



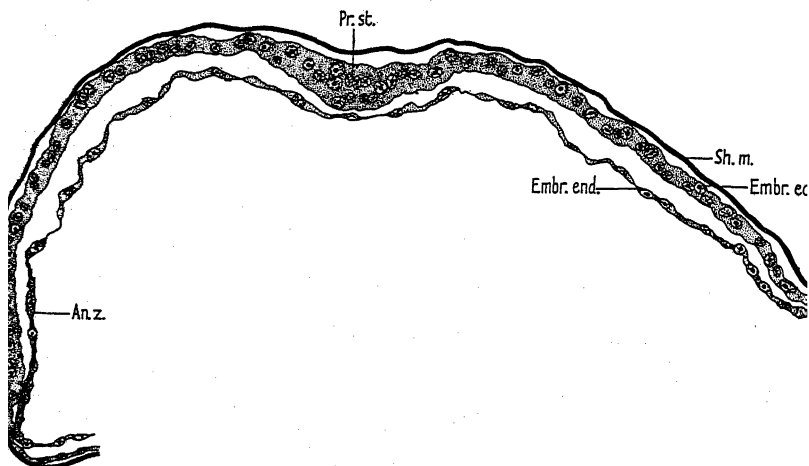
TEXT-FIG. 1.

Embryonal area of Stage I (1.32 mm. and 0.99 mm.). *Pr. st.*, primordium of primitive streak.  $\times 30$ .

(1927) on the Pig, have shown that in these Mammals the primitive streak makes its appearance at the margin of the embryonal area, so that if this thickening in *Bettongia* is indeed the primordium of the primitive streak its position is quite unusual. The thickening contains fifteen or twenty more nuclei than would a corresponding piece of ordinary embryonal ectoderm. So far no mesoderm has become separated off as individual cells, but this is apparently about to begin (Text-fig. 2; fig. 6, Pl. 41). There is already present, round the periphery of the embryonal region, the zone of thickened endoderm which becomes a source of mesoderm formation in later stages, and which corresponds to the annular zone of proliferation of Hubrecht (1890) and the 'Mesoblasthof' of Bonnet (1884). It is noticeable that this zone appears here very much earlier than in *Sorex*. It can be seen (Text-fig. 2) as a region of endoderm, about 0.15 mm. broad, in which the nuclei are more numerous and the cytoplasm more abundant and more granular than else-



where in the endoderm; at this early stage it is not everywhere so clearly defined as it is later, and it is not yet active. Its relative position changes as growth proceeds; here it can be seen to lie almost entirely under the edge of the embryonal ectoderm, later it is barely overlapped by the latter. In these



TEXT-FIG. 2.

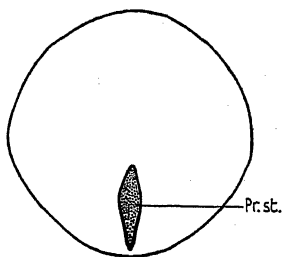
Transverse section of Stage I (1.32 mm. and 0.99 mm.). *An. z.*, annular zone; *Embr. ect.*, embryonal ectoderm; *Embr. end.*, embryonal endoderm; *Pr. st.*, primitive streak primordium; *Sh. m.*, shell-membrane; *X.*, edge of embryonal area.  $\times 200$ .

early stages the curvature of the blastocyst makes it impossible to be sure from transverse sections if the breadth of this zone is equal all round. No trace can yet be detected of a prochordal plate. At three places in the embryonal area there is present between ectoderm and endoderm an apparently normal cell, the origin of which is doubtful. A shell-membrane is present and is about  $3\mu$  thick; it is also present in all the other blastocysts described, becoming thinner in an irregular manner in later stages.

Stage II (diameter 1.40 mm.).

Stage II follows very closely upon Stage I, but the series of sections is not quite complete. The primitive streak primordium

has here extended centrally and peripherally to form a linear streak (Text-fig. 3). This stage is of importance in that it indicates that the position of origin of the streak in the last stage is not abnormal, for if the broadest point of this developing



TEXT-FIG. 3.

Embryonal area of Stage II (1.40 mm.). *Pr. st.*, primitive streak.  
× 30.

streak is taken as the point of its first appearance, it is found to be in very nearly the same position as in Stage I. The streak is 0.34 mm. in length. A number of mesoderm cells have by



TEXT-FIG. 4.

Transverse section of streak and half embryonal area of Stage II (1.40 mm.). *An. z.*, annular zone; *Mes.*, mesoderm; *Pr. st.*, primitive streak. × 185.

now become dissociated from the streak and have migrated into the space between ectoderm and endoderm (Text-fig. 4); posteriorly they extend to within a few sections of the edge of the embryonal area, but elsewhere they do not extend far. The streak has still not reached either the centre of the embryonal area or its periphery, so that there is as yet no sign of primitive knot or of caudal broadening. Nor has a primitive groove developed, although in some sections about the middle there are indications of its appearance. The annular zone of endoderm

is distinct, and about 0.18 mm. broad; it is only partially overlapped by the embryonal ectoderm. There is no prochordal plate.

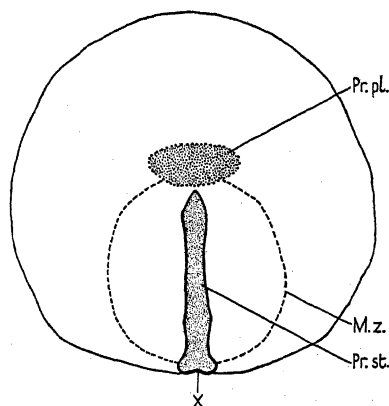
Stage III (diameter 1.60 mm.).

Longitudinal sections were made of this stage, which is not however in a good state of preservation. Considerably more mesoderm has been produced, and the streak has increased in length so that it now reaches to the hinder margin of the embryonal area. There does not appear to be any prochordal plate.

Stage IV (diameter 1.88 mm.).

In this blastocyst the outlines of the embryonal area and primitive streak could be seen from external examination by transmitted light, although still with difficulty. The embryonal area is circular, 1.52 mm. in diameter, and the streak is 0.74 mm. in length, so that it stretches almost to the centre of the area. At its posterior end the streak tissue broadens out and bifurcates, the hinder extremities of the bifurcation terminating at the margin of the embryonal area; this bifurcation is found in a more or less pronounced form in the immediately succeeding blastocysts and apparently represents the caudal knot (Text-fig. 5). The formation of a primitive knot has not yet commenced. Down the centre of the streak the primitive groove is making its appearance, in a rather irregular manner; in the posterior third of the streak it appears as a deep cleft, more anteriorly it flattens out into a shallow groove, then deepens again into the condition seen in Text-fig. 6, and in the anterior third it is no longer present, leaving the streak as a simple thickening of the ectoderm. The groove is irregularly shaped in transverse section, and may appear double; possibly it was some appearance of this kind that is figured by Selenka (1892, fig. 10, Pl. xxxii). Altogether the groove extends through almost two-thirds of the streak, but its presence does not seem to be correlated in any way with the amount of mesoderm being produced. Active proliferation of mesoderm is now occurring from the sides of the streak throughout most of its length; towards either

end it slackens off, ceasing a little short of the edge of the embryonal area posteriorly, and at the anterior end becoming desultory. The lack of mesoderm posterior to the streak may



TEXT-FIG. 5.

Embryonal area of Stage IV (1.88 mm.). *M. z.*, edge of mesoderm zone; *Pr. pl.*, prochordal plate; *Pr. st.*, primitive streak; *X*., posterior 'bifurcation' of streak.  $\times 30$ .



TEXT-FIG. 6.

Transverse section of streak and half embryonal area of Stage IV (1.88 mm.). *An. z.*, annular zone; *Pr. st.*, primitive streak.  $\times 120$ .

perhaps, as indicated by both earlier and later stages, be an individual peculiarity of this blastocyst. The mesoderm has not yet extended very far laterally, it occupies an oval area, approximately 0.8 mm. by 0.7 mm., though isolated cells are present beyond these limits (Text-figs. 5 and 6; fig. 9, Pl. 42). The annular zone is distinct, and laterally measures about

0.25 mm. It is certainly not producing mesoderm to any marked extent, but occasionally there can be seen overlying it mesoderm cells which are about twice as far away from the streak as their nearest neighbours and which may therefore have been produced by this endoderm. A search was made for mitotic figures with their axes of division so arranged that the completion of the division would with certainty have produced mesoderm cells (as figured by Hubrecht, 1890, in *Sorex*), but in this series such appearances were not found; there remains the possibility that mesoderm is produced by the emigration of cells from this zone in a manner comparable to the emigration of the endoderm mother cells. This question will be discussed later. In front of the primitive streak the endoderm is thickened slightly into a prochordal plate (fig. 10, Pl. 42); in later stages this plate is still present but is more difficult to define owing to the closely investing mesoderm which lies upon it. It is oval in shape and measures about 0.38 mm. by 0.16 mm.; at present it is not in an active state, judging by the scarcity of mitotic figures, so possibly the few mesoderm cells overlying it have not been produced in situ but are merely the most anterior of those migrating forwards from the primitive streak. An increase in the thickness of the extra-embryonal as compared with the embryonal endoderm has taken place, and one effect of this is to make the inner edge of the annular zone much easier to determine than the outer.

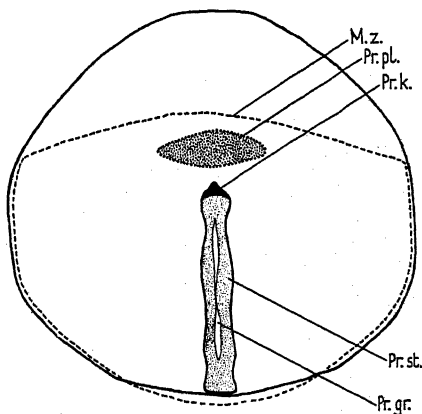
#### Stage V (diameter 2.07 mm.).

This was a crumpled and slightly damaged blastocyst, whose true diameter was probably considerably greater than that given. By transmitted light the primitive streak showed as a dark line, 1.13 mm. in length, with a slightly broader anterior end, stretching from the edge of the lighter embryonal area. Active production of mesoderm is going on from the whole length of the streak; near to the streak itself the sheet is several cells thick, but this thins out gradually towards the edge of the area. Mesoderm cells overlying the annular zone, some within the embryonal area and some without it, are frequent, and a considerable number are to be found posterior to the streak.

It is not possible to describe a prochordal plate as the sections of this region are not satisfactory.

Stage VI (diameters 2.23 mm. and 2.13 mm.).

This stage shows in surface view the dark streak, 0.96 mm. in length, stretching more than half-way along the oval embryonal area, which measures 1.63 mm. by 1.49 mm. (fig. 1, Pl. 41). In



TEXT-FIG. 7.

Embryonal area of Stage VI (2.23 mm. and 2.13 mm.). *M. z.*, mesoderm zone; *Pr. gr.*, primitive groove; *Pr. k.*, primitive knot, with short head-process; *Pr. pl.*, prochordal plate; *Pr. st.*, primitive streak.  $\times 30$ .

sections the primitive groove is still very irregular in shape, showing in some places as a deep cleft and in others shallowing off almost to disappearance. Mesoderm is being produced all along the sides of the primitive streak and shows as a sheet of cells thinning out towards the periphery; laterally its boundary is just short of the edge of the embryonal area, though a few cells occur in extra-embryonal positions. At the hinder end of the area the mesoderm continues round the posterior end of the streak in considerable quantity; there are no manifestations of great activity in the annular zone in this region and the mesoderm is quite continuous with the peristomal sheets, so presumably part of it at least is of primitive streak origin (Text-fig. 7). The primitive knot is not so well marked

as in later stages, but is thicker than the streak and broader than that part of the streak immediately behind it; no definite blastoporic depression can be made out. Stretching forwards in the axial line from the primitive knot is the primordium of the head-process; in this series its anterior end is separated by a considerable space from the prochordal plate, and it is quite continuous with the mesoderm to either side (fig. 11, Pl. 42). The oval prochordal plate is present a little in front of the head-process; it appears to be producing a certain amount of mesoderm, but the precise amount is obscured by the fact that cells appear to be migrating forwards over it from the anterior end of the primitive streak. A section through the margin of the plate is shown in fig. 15, Pl. 42, and it can be seen that it does not consist of a sharply delimited plate several cells thick but rather of an irregularly thickened area one or occasionally two cells in thickness, in which it is often hard to say whether particular cells are part of the plate itself or are mesoderm cells closely apposed to it. The mesoderm present laterally and anteriorly to the plate, as indicated by the broken line in Text-fig. 7, is very scanty. The annular zone is laterally about 0.28 mm. broad.

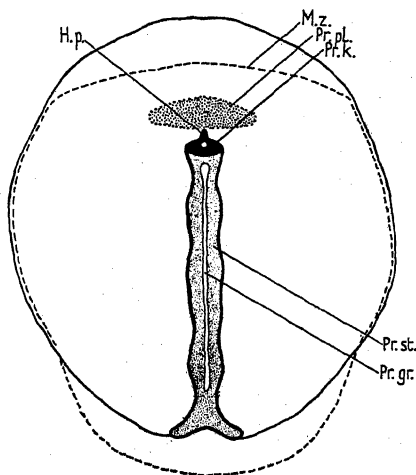
Stage VII (diameters 2.76 mm. and 1.97 mm.).

This was a very collapsed blastocyst in which no details at all were visible externally. Since it is very rare for a groove in the blastocyst wall to pass through the embryonal area, presumably owing to its greater thickness, the specimen was cut on the assumption that its flattest side was embryonal; this proved to be correct and the sections turned out to be almost longitudinal. The embryonal area appears to be circular, about 1.50 mm. in diameter; the primitive streak is 0.89 mm. in length. The streak itself is normal in appearance; posteriorly it broadens out somewhat as it reaches the edge of the area, and at its anterior end it is both broader and thicker than in the middle, presumably this represents a developing primitive knot but neither blastoporic depression nor head-process could be distinguished. Abundant mesoderm is being produced laterally and a little occurs anteriorly, but posteriorly there are only

isolated cells. A prochordal plate, showing little activity, is present. In this blastocyst the anterior and posterior portions of the annular zone can be compared directly; the posterior measures about 0.24 mm. in breadth and is well formed, the anterior is only slightly less broad but is not nearly so distinctly marked.

Stage VIII (diameter 3.76 mm.).

In this very well preserved blastocyst the dark primitive streak with its posterior 'bifurcation' shows up distinctly by



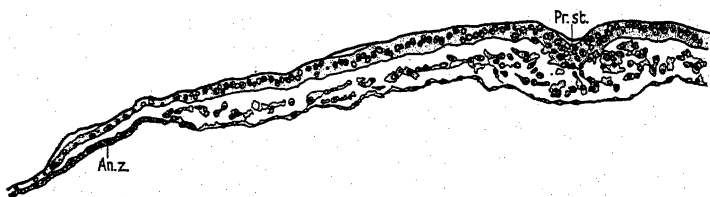
TEXT-FIG. 8.

Embryonal area of Stage VIII (3.76 mm.). *H. p.*, head-process; *M. z.*, mesoderm zone; *Pr. gr.*, primitive groove; *Pr. k.*, primitive knot, with blastoporic depression; *Pr. pl.*, prochordal plate; *Pr. st.*, primitive streak.  $\times 30$ .

transmitted light against the lighter embryonal area (fig. 2, Pl. 41). Round the margin of the embryonal area there is a still lighter zone, also noticed in some other blastocysts, which apparently represents the gap between the edge of the embryonal area and the line where ectoderm and endoderm actually come into contact (Text-fig. 9). The oval embryonal area measures 1.97 mm. by 1.62 mm.; the length of the streak and



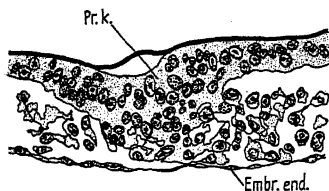
knot combined is 1.24 mm., and that of the streak alone about 1.15 mm. Up the centre of the streak runs the well-formed primitive groove (Text-figs. 8 and 9); throughout its length it



TEXT-FIG. 9.

Transverse section of streak and half embryonal area of Stage VIII (3.76 mm.). *An. z.*, annular zone; *Pr. st.*, primitive streak.  $\times 70$ .

varies considerably in depth, but on the whole it is shallower towards its anterior end. It completely flattens out just before the tissue of the streak becomes merged into that of the knot.



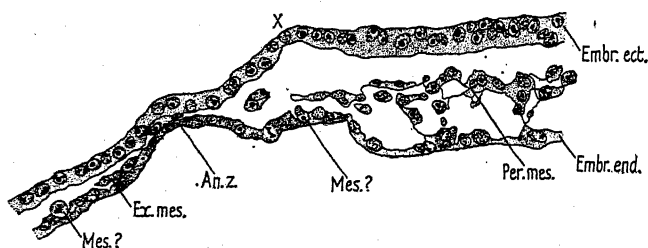
TEXT-FIG. 10.

Transverse section of primitive knot of Stage VIII (3.76 mm.). *Embr. end.*, embryonal endoderm; *Pr. k.*, primitive knot, with blastoporic depression.  $\times 175$ .

The knot is much thicker in transverse section (Text-fig. 10, and fig. 13, Pl. 42) than the streak (Text-fig. 9, and fig. 14, Pl. 42), and also somewhat broader; it is of a looser consistency and as a result rather lighter in appearance. On its dorsal surface, and well separated from the primitive groove, there is a shallow circular blastoporic depression, about 0.04 mm. in diameter and about 0.02 mm. in greatest depth; one edge of this depression is shown in Text-fig. 10, and fig. 13, Pl. 42. Under the knot the endoderm is consistently thin and in places

makes slight contacts with the knot tissue as in Text-fig. 10, but nowhere is there any fusion between the two at this stage. The head-process is still not greatly developed; it extends forwards from the knot to about the edge of the prochordal plate. The peristomal mesoderm is continuous in front with a sheet of mesoderm which extends laterally round the sides of the knot and head-process and passes forwards over the prochordal plate; fig. 12, Pl. 42, represents a transverse section just posterior to the edge of the plate. In it also can be seen how the mesoderm thins out laterally; this thinning out occurs in a similar manner anteriorly, so that there is but little mesoderm over the anterior part of the prochordal plate. The peristomal mesoderm extends from either side of the streak to the margin of the embryonal area (Text-fig. 9). Just beyond the edge of the embryonal area, laterally to the primitive streak, extra-embryonal mesoderm is found as isolated cells and as small groups of cells, and posterior to the streak it occurs in considerable quantities; this posterior mesoderm passes without a break into the peristomal mesoderm, and is probably derived in part from the annular zone and in part from the primitive streak. As already mentioned, a certain amount of mesoderm is being produced by the annular zone from an early stage. The precise amount is hard to estimate; first, because such cells may arise by the migration of complete cells, as in endoderm formation itself, as well as by the much more easily detectable process of mitotic division, and second, because in preparations, in which the endoderm is almost certainly to some extent altered out of its original state, appearances occur which might be interpreted as examples of both methods, but of whose genuineness it is difficult to be certain. Nevertheless, cases of cells apparently in the process of emigration (Text-fig. 11) or of mitosis (fig. 8, Pl. 41) which would give rise to mesoderm are sufficiently common to make it clear that the endoderm of the annular zone does actually give rise to mesoderm. The activity of the zone, judged both by the total number of mitotic figures and by the amount of mesoderm, dies away as its anterior quarter is approached. The zone is about 0.35 mm. broad laterally, and is only just overlapped by the embryonal ectoderm. The prochordal plate shows by its

numerous mitotic figures that it is in a condition of considerable activity; here again the same difficulty arises as with the annular zone, and all that can be said with certainty is that a contribution of mesoderm is being made. The plate is still in general but one cell thick, but, though distinctly thicker than the surrounding endoderm when characteristic portions of the two are compared, its boundaries are rendered difficult to define in places owing to the overlying mesoderm which closely invests



TEXT-FIG. 11.

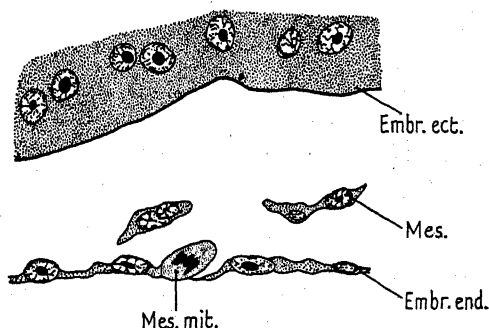
Transverse section of edge of embryonal area of Stage VIII (3.76 mm.). *An. z.*, annular zone; *Embr. ect.*, embryonal ectoderm; *Embr. end.*, embryonal endoderm; *Ex. mes.*, extra-embryonal mesoderm; *Mes?*, mesoderm cell about to be produced?; *Per. mes.*, peristomal mesoderm; *X*, edge of embryonal area.  $\times 185$ .

the endoderm throughout most of this region. Anteriorly and laterally to the plate mesoderm cells occur in decreasing numbers as far forward as the boundary shown (Text-fig. 8). In this blastocyst three cases have been noticed in which cells of the general embryonal endoderm are about to give rise to mesoderm cells by mitosis; the dividing cell in these cases, as shown in Text-fig. 12, has partially freed itself from the surrounding endoderm cells before commencing to divide, and the orientation of the mitotic figure itself, though of the plane of the endoderm is not at right angles to it, as is usual with the mesoderm-producing mitoses of the prochordal plate and annular zone (fig. 8, Pl. 41). Endoderm cells in such a position, namely arranged with one end of the cell in the endoderm layer but with the bulk of their substance lying in the space between ectoderm and endoderm, are not uncommon, but whether or

not they are important as a source of mesoderm cannot be determined from the present material.

Stage IX (diameter 4.40 mm.).

This specimen is shown in fig. 3, Pl. 41, in which several details of its structure can be made out. The embryonal area is elongated and slightly pear-shaped, 3.10 mm. by 2.20 mm.



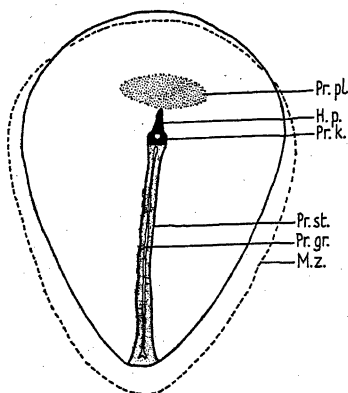
TEXT-FIG. 12.

Transverse section of part of embryonal area of Stage VIII (3.76 mm.). *Embr. ect.*, embryonal ectoderm; *Embr. end.*, embryonal endoderm; *Mes.*, mesoderm; *Mes. mit.*, mitosis in endoderm about to produce a mesoderm cell.  $\times 450$ .

in maximum length and breadth respectively; from its posterior margin and along its long axis stretches the primitive streak, 1.92 mm. in length (including the knot), and therefore still distinctly more than half the length of the area itself. At its posterior end the streak expands into a dark and somewhat sickle-shaped area; this appearance recalls Bonnet's (1897) figure for the Dog, but in sections it is found to be largely the result of condensation in the mesoderm. At its anterior end the streak ends in a small dark cap, the primitive knot. The innermost, thickest part of the peristomal mesoderm shows as an opacity on either side of the streak, and the opacities are continued forward in front of the knot as two small dark projections, one on either side of the light head-process region. Outside the embryonal area could be seen, in its lateral and posterior extent, the edge of the expanding mesodermal sheet, and beyond

this again appeared the line where ectoderm and endoderm come into contact; these two points cannot be made out from the photograph. In the posterior third of the streak the primitive groove could be seen as a lighter line, ending posteriorly in a distinct bifurcation after the manner of the earlier blastocyst figured by Selenka (1892).

Sections show that mesoderm production from the posterior



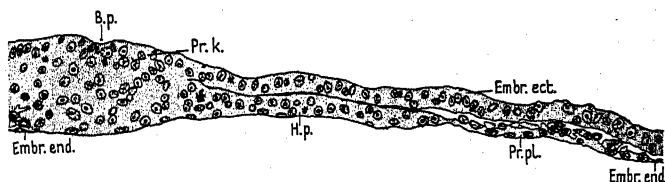
TEXT-FIG. 13.

Embryonal area of Stage IX (4-40 mm.). *H. p.*, head-process; *M. z.*, mesoderm zone; *Pr. gr.*, primitive groove; *Pr. k.*, primitive knot, with blastoporic depression; *Pr. pl.*, prochordal plate; *Pr. st.*, primitive streak.  $\times 15$ .

part of the streak is proceeding irregularly, and the mesoderm cells themselves are spreading laterally and to a small extent posteriorly. At the anterior end of the streak is the primitive knot, with a structure similar to that described in the last blastocyst, including the small blastoporic depression. The head-process is well established (Text-fig. 14) as a solid rod of cells stretching forward from the anterior face of the knot; it is about 0.20 mm. in length, but there is no sign of a lumen. Mesoderm is being actively produced by the primitive streak, and now exists as a sheet extending beyond the embryonal area laterally and posteriorly; anteriorly it reaches unbroken almost to its edge (Text-fig. 13). The text-figure just referred to was

constructed partly from a graph of the sections and partly from a drawing of the complete blastocyst.

As in the preceding blastocysts the annular zone and prochordal plate are distinct from each other, there is never any fusion of the two as occurs at an early stage in *Manus* (van Oordt, 1921); as before also both these areas are in an active state and making a contribution to the mesoderm. Anteriorly and posteriorly the breadth of the annular zone is similar, about 0.38 mm., but even now there is little mesoderm overlying its extreme anterior end. Under the knot and head-process the endoderm is not discernible as a separate layer (Text-fig. 14). Continuous with either side of the process is a thick sheet of



TEXT-FIG. 14.

Longitudinal section of knot and head-process in Stage IX (4.40 mm.). *B. p.*, blastoporic depression; *Embr. ect.*, embryonal ectoderm; *Embr. end.*, embryonal endoderm; *H. p.*, head-process; *Pr. k.*, primitive knot; *Pr. pl.*, prochordal plate, with overlying mesoderm.  $\times 170$ .

mesoderm, and at its tip the process merges into the edge of the prochordal plate. The plate shows numerous mitoses, but as before the overlying mesoderm makes its boundary often hard to define.

Stage X (diameter about 5.5 mm.).

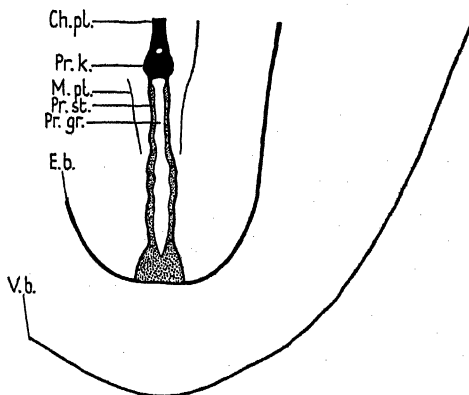
The uterus was fixed entire in Carnoy's fluid, and then cut open with a safety razor blade and preserved in absolute alcohol. During the incisions of the uterus the blastocyst was cut into four separate pieces, but owing to the disposition of the cuts and the straightness of their edges it was possible to reconstruct the complete blastocyst with a fair degree of precision. The diameter was apparently about 5.5 mm. The embryonal area was elongated, about 3.85 mm. in length, and constricted in the

middle, being about 1.52 mm. broad in the region of the primitive knot, then narrowing to about 1.25 mm., and finally broadening out again to about 1.72 mm. in front. The general condition of the embryo was good. Only one cut passed through the embryonal area, along a slanting line a little in front of the knot. The segment posterior to the cut (fig. 4, Pl. 41), which contains the primitive streak and knot and the beginning of the head-process, may first be described, followed by a brief description of the anterior segment (fig. 5, Pl. 41).

Viewed by transmitted light the streak showed as a light line with dark edges, about 1.68 mm. in length (to the middle of the knot thickening), the dark edges marking the margins of the primitive groove. At its posterior end the streak darkened and broadened out, without any sign of 'bifurcation'; at its anterior end appeared the broader, darker primitive knot, from which the head-process extended forward as a light line. Between the outline of the embryonal area and that of the streak was visible a broad dark line representing the longitudinal thickened band of mesoderm of that side; beyond that appeared the margin of the embryonal area, and much farther out still appeared the line where ectoderm and endoderm now come into contact. The blood-vessels developing in the vascular mesoderm which lies between these last two boundaries could be clearly seen. Most of these details can be made out in the photograph (fig. 4, Pl. 41).

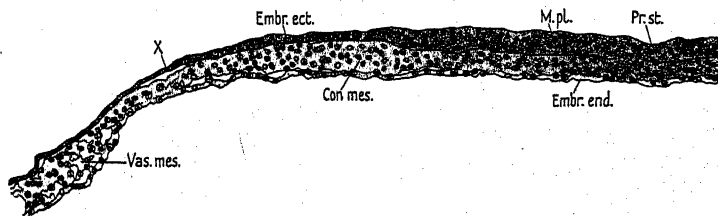
In sections the primitive streak is broad and rather flattened (Text-fig. 16), posteriorly it expands somewhat, and anteriorly its tissue merges into that of the primitive knot (Text-fig. 15). Mitotic figures are not common in its substance, very much less so than in earlier stages, and it seems possible that it is now producing comparatively little mesoderm, the stage having been reached when the bulk of the new mesoderm is produced by divisions of pre-existing mesoderm cells. The great breadth of the primitive groove is noticeable; it deepens rapidly from its posterior end but soon shallows into the condition shown in Text-fig. 16. The knot as before is rather bulkier in section than the streak and lighter staining; on its surface a small blastoporic depression is visible, and the head-process extends forwards

from its anterior face. The ectodermal thickening forming the medullary plate extends far back on either side of the streak,



TEXT-FIG. 15.

Embryonal area, posterior portion, of Stage X ('5.5' mm.). *Ch. pl.*, chorda plate; *E. b.*, boundary of embryonal area; *M. pl.*, medullary plate; *Pr. k.*, primitive knot and blastopore depression; *Pr. gr.*, primitive groove; *Pr. st.*, primitive streak; *V. b.*, boundary of vascular area.  $\times 20$ .



TEXT-FIG. 16.

Transverse section of streak and half of embryonal and vascular areas of Stage X ('5.5' mm.). *Embr. ect.*, embryonal ectoderm; *Embr. end.*, embryonal endoderm; *M. pl.*, medullary plate; *Con. mes.*, Condensed band of mesoderm; *Pr. st.*, primitive streak; *Vas. mes.*, vascular mesoderm; *X*, edge of embryonal area.  $\times 90$ .

the ectoderm being distinctly thicker and its nuclei tending to be arranged with their long axes at right angles to the surface (Text-fig. 16). The amount of mesoderm present has increased enormously and has spread far beyond the limits of the

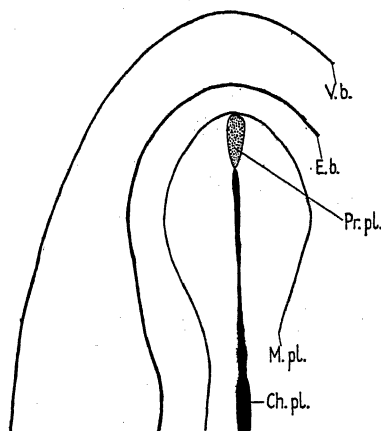


embryonal area. Within the embryonal area it has concentrated to some extent into two longitudinal bands, disposed distally to the margins of the medullary plate (Text-fig. 16), and it is noticeable how much commoner are mitoses in these bands than elsewhere. The peripheral meeting of ectoderm and endoderm, instead of being just beyond the margin of the embryonal area, is removed far outwards, and the space between them is occupied by a large amount of mesoderm. In this mesoderm are appearing spaces of various shapes and sizes, many of which are lined with a definite endothelium and are clearly developing blood-vessels (fig. 17, Pl. 42); these vessels may stretch through a number of sections, but free blood-cells or blood-islands are never present in their lumina. Between the vessels, however, can be seen groups of cells, showing well in the above Plate, and it seems likely that these later give rise to blood-cells by migrating into the vessels through their walls. This vascular zone appears quite distinctly in the photographs of the blastocyst (figs. 4 and 5, Pl. 41); it surrounds the whole embryonal area, but vessel formation is most advanced in the portions at about the level of the primitive knot. The largest vessels appear, in sections, about the middle of the vascular area; there is no *sinus terminalis*.

The endoderm under the streak is closely apposed to the mesoderm but is not fused with it, and can be distinguished from it by its lighter appearance (Text-fig. 16); with the knot, however, fusion does occur, the endoderm appearing to pass into the lower edges of the knot tissue. It will be noticed in the last Text-figure that the embryonal endoderm has greatly increased in thickness; this also applies to the endoderm underlying the vascular area, and possibly because of this or possibly because it has ceased to function, the annular zone can no longer be identified.

The knot tissue prolongs itself forwards for some little distance under the ectoderm, and is little diminished when the medullary groove makes its appearance above it; but gradually it thins and narrows into a chorda plate a few cells thick, and at this point the cut edge of the posterior segment of the embryonal area is reached. In the hinder part of the anterior segment (Text-fig. 17)

the chorda plate flattens out still more to become a plate five or six cells (80 or 100 $\mu$ ) broad and one, or occasionally two, cells thick; the plate is continuous with the endoderm at either side, but its cells are somewhat columnar in shape and larger and more deeply staining than those of the endoderm. No mesoderm is present between the plate and the ectoderm of the medullary

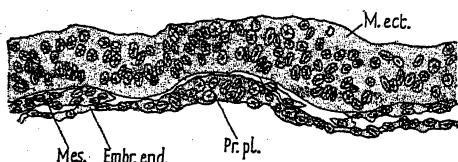


TEXT-FIG. 17.

Embryonal area, anterior portion, of Stage X ('5.5' mm.). *Ch. pl.*, chorda plate; *E. b.*, boundary of embryonal area; *M. pl.*, medullary plate; *Pr. pl.*, prochordal plate; *V. b.*, boundary of vascular area.  $\times 20$ .

groove, indeed the two are often very closely apposed; the mesoderm sheet commences at either edge of the plate and is sometimes attached to it. In the middle third of its extent in this segment, the chorda plate, after varying somewhat in breadth and being in places quite poorly developed, thickens into the chorda proper, a typical section of which is shown in fig. 7, Pl. 41. This part of the structure, shown thinnest in Text-fig. 17, is usually three cells broad and two cells thick. It is often in close contiguity with the ectoderm of the medullary groove but is distinctly separated from the mesoderm. The endoderm appears to fuse with its lower edges and while in places it might be continuous below it, this nowhere shows clearly. In this region too the medullary groove is best developed,

reaching about  $30\mu$  in depth; anteriorly it flattens out to a great extent but can be distinguished as a slight groove floored by thinner ectoderm. In its anterior third the chorda thins and broadens out a little into a plate four or five cells in width; in appearance this is much like its posterior third except that the endoderm shows a tendency to encroach under its edges. The shape and greater granularity of its cells generally make them easy to distinguish from the endoderm, except for a few sections at its extreme anterior end. This is due to the fact that its tip



TEXT-FIG. 18.

Transverse section of prochordal plate of Stage X ('5.5' mm.).

*Embr. end.*, embryonal endoderm; *M. ect.*, medullary ectoderm;  
*Mes.*, mesoderm; *Pr. pl.*, prochordal plate.  $\times 200$ .

is continuous with the prochordal plate. The plate is now represented by an oval thickened area of endoderm (Text-fig. 17) lying entirely under the anterior end of the brain-plate; the cytoplasm of its cells stains slightly darker than that of the surrounding endoderm but they lack the slightly columnar appearance of the chorda plate cells and its lateral margins are not so sharply demarcated (Text-fig. 18). It increases in thickness towards its anterior margin, where it is several cells thick. Here and there free mesoderm cells occur between it and the brain-plate, but are not numerous; at its edges it frequently appears continuous with the mesoderm sheets which meet in the mid-line round its anterior margin. Over the posterior part of the plate there is a secondary deepening of the medullary groove, which shallows out anteriorly.

The mesodermal sheet in the anterior segment of the embryonal area is differentiated into the same three parts as farther back; from the axial line to just beyond the margin of the medullary plate there is a uniform layer about three cells thick and not very dense, then comes the much denser con-

densation and finally the vascular mesoderm; the first two parts are less dense than in the primitive streak region, and become slightly less developed as one passes forwards, but both are continuous in the embryonal area round the anterior end of the brain-plate. At the level where the chorda is best formed, and shown thinnest in Text-fig. 17, and where the edges of the medullary plate are becoming raised above the embryonal ectoderm, the pleuro-pericardial coelom is making its appearance in the longitudinal condensations of the mesoderm. It appears on either side as a number of irregular spaces and farther forward these unite into one or two larger spaces, as shown in fig. 16, Pl. 42. More anteriorly still, the coelom narrows down again into a slit-like space or spaces, sometimes barely distinguishable, those of the two sides becoming continuous round the anterior end of the brain-plate.

#### DISCUSSION.

The first appearance of the primitive streak as a localized thickening of the ectoderm situated about mid-way between the centre of the embryonal area and its periphery, as appears to be the case in *Bettongia*, has so far not been observed in any other Mammal. There are only two blastocysts in the present material with a bearing on this point, but at present no other Marsupial material of this stage appears to have been examined. That the bulk of the mesoderm in *Bettongia* arises from the primitive streak is quite clear, but the amount produced elsewhere is extremely difficult to estimate. Certainly there is a contribution from the prochordal plate and the annular zone as described. The question, however, of mesoderm formation by the emigration of complete cells out of the endoderm cannot be settled by this type of investigation when such cells do not give a differential staining reaction, but that the possibility of this emigration should not be dismissed too lightly is indicated by the obvious difficulty which would have been encountered in understanding endoderm formation but for the eosinophil nature of the endoderm mother cells. At present it seems necessary to suppose, as suggested by Hill (1910) for endoderm formation, that the two processes of mitosis and of

emigration may be occurring together. There are also the 'mesoderm' cells seen in the first stage to be accounted for as well as the contribution from the general embryonal endoderm seen in the eighth stage.

The prochordal plate is poorly developed in *Bettongia*, making its study difficult, but that this is not a universal feature in Marsupials is shown by the well-developed prochordal plate of *Perameles obesula* (Wilson and Hill, 1907). The material at our disposal is lacking firstly in stages which might demonstrate a connexion between prochordal plate and annular zone, if indeed such ever develops, and secondly in stages which might show whether the annular zone plays any part in the early development of the vascular mesoderm.

The condition of the vascular tissue in the last stage is most interesting as the vessels appear to be forming without any contained blood-islands, and from the sections it certainly appears as if the blood-islands are represented by the groups of cells situated outside the blood-vessels. In this instance again more material is necessary to establish the mechanism of the process involved.

#### SUMMARY.

1. A series of ten blastocysts of *Bettongia cuniculus* is described, covering a range of development from the appearance of the primitive streak to the establishment of the medullary plate.

2. In the first blastocyst there has appeared a small area of ectodermal thickening between the centre of the embryonal area and its periphery, and it is suggested that this is the primordium of the primitive streak. In the second blastocyst a very young streak is present which does not reach either to the middle or to the periphery of the area, and whose centre is in nearly the same position as that of the first blastocyst. At its maximum the streak stretches about two-thirds of the way across the embryonal area. A groove develops down its middle and active mesoderm production occurs from its edges. The mesoderm forms an approximately oval area round the streak, and extends into an extra-embryonal position first posteriorly and then laterally.

3. At the anterior end of the streak appears a broader, thicker region of similar but looser tissue, the primitive knot; from here grows forward the head-process in the usual way, and at the same time the endoderm underlying these two structures fuses with them. No lumen is present in the head-process at any stage. The originally circular embryonal area elongates until it becomes somewhat pear-shaped, and later it constricts in the middle.

4. The thickened ring of endoderm at the edge of the embryonal area, the annular zone of proliferation of Hubrecht, is distinguishable at the time of appearance of the primitive streak; but the second thickened area, the prochordal plate, does not appear until the streak is well established, and except in its earliest stages is hard to delimit owing to its relative thinness and to the closely investing mesoderm which soon covers it. The question of mesoderm production from these sources is discussed, and it is concluded that while such does in fact occur in this form it is quite insignificant in amount as compared with that produced from the sides of the primitive streak.

5. In the last stage described the medullary plate and chorda are well established, and the importance of the primitive streak is diminishing. Mesoderm, whose production now appears to be occurring chiefly by division of pre-existing mesoderm cells, is very much more abundant, and can be divided into three parts: thick sheets extending from near the axial line to beyond the edge of the medullary plate, condensations in which the pleuro-pericardial coelom is appearing anteriorly, and vascular mesoderm in which blood-vessels are forming. Blood-islands are not present within the vessels, and apparently are represented by groups of cells between the vessels. A well-formed prochordal plate is also present.

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## EXPLANATION OF PLATES 41 AND 42

### PLATE 41.

- Fig. 1.—Surface view (by transmitted light) of Stage VI (2.23 mm. and 2.13 mm.).  $\times 17\frac{1}{2}$ .
- Fig. 2.—Surface view of Stage VIII (3.76 mm.).  $\times 17\frac{1}{2}$ .
- Fig. 3.—Surface view of Stage IX (4.40 mm.).  $\times 16\frac{1}{2}$ .
- Fig. 4.—Surface view of Stage X ('5.5' mm.), posterior segment.  $\times 13$ .
- Fig. 5.—Surface view of Stage X ('5.5' mm.), anterior segment.  $\times 13$ .
- Fig. 6.—Transverse section of primordium of primitive streak of Stage I (1.32 mm. and 0.99 mm.).  $\times 250$ .
- Fig. 7.—Transverse section of medullary groove and chorda of Stage X ('5.5' mm.).  $\times 250$ .
- Fig. 8.—Transverse section of edge of annular zone of Stage VIII (3.76 mm.) showing a mitosis which will give rise to a mesoderm cell.  $\times 375$ .

### PLATE 42.

- Fig. 9.—Transverse section of streak and half of embryonal area of Stage IV (1.88 mm.) showing primitive streak, mesoderm, and edge of annular zone.  $\times 115$ .
- Fig. 10.—Transverse section of prochordal plate of Stage IV (1.88 mm.).  $\times 115$ .
- Fig. 11.—Transverse section of early head-process of Stage VI (2.23 mm. and 2.13 mm.).  $\times 175$ .

Fig. 12.—Transverse section of extreme anterior end of head-process of Stage VIII (3.76 mm.).  $\times 115$ .

Fig. 13.—Transverse section of primitive knot of Stage VIII (3.76 mm.).  $\times 200$ .

Fig. 14.—Transverse section of primitive streak of Stage VIII (3.76 mm.).  $\times 200$ .

Fig. 15.—Transverse section of edge of prochordal plate of Stage VI (2.23 mm. and 2.13 mm.).  $\times 375$ .

Fig. 16.—Transverse section of edge of medullary plate and pleuro-pericardial coelom of Stage X ('5.5' mm.). *M.f.*, medullary fold; *P.-p.c.c.*, pleuro-pericardial coelom; *Sh.m.*, shell-membrane.  $\times 250$ .

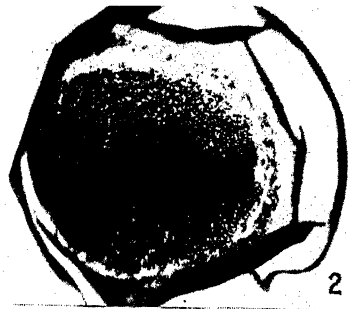
Fig. 17.—Transverse section of part of vascular area of Stage X ('5.5' mm.). *B.i.*, 'blood-islands'; *B.v.*, blood-vessel; *Ect.*, ectoderm; *End.*, endoderm.  $\times 250$ .



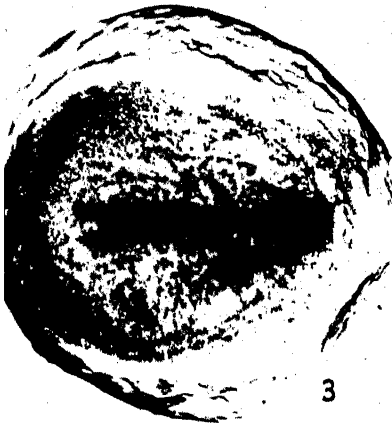




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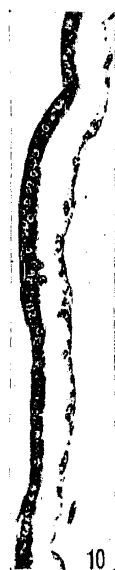
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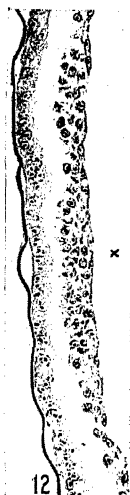
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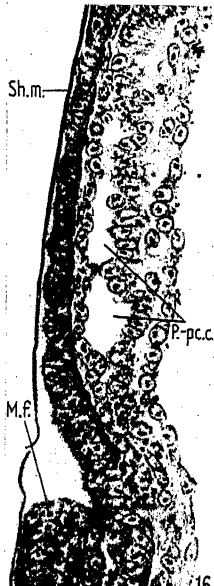
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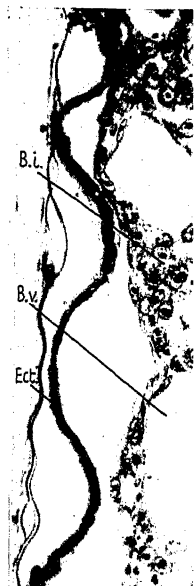
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